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ABSTRACT OF THE THESIS

The Effects of Diet Calcium, Protein and Acidity on Calcium Retention in the Rat, Particularly as Related to the Inner and Outer Surfaces of Tubular Bone: A Possible Model for th Treatment or Prevention of Osteoporosis

by JOSEPH E. MILLIGAN, D.V.M., Ph.D.

Thesis Director: Professor Joseph Liston Evans

The effects of graded levels of diet calcium, protein and acidity on radiographic and gravimetric measurements, and on mineral composition of femurs were studied in Long Evans, young and old, female rats.

Calcium depletion produces osteopenia ("osteoporosis") in the young rat, through the mechanisms of increased bone resorption and decreased deposition, while calcium repletion overcomes this "osteoporotic" condition through increased bone deposition and decreased resorption. Increasing the repletion diet calcium level from 0.22 to 0.78% improves the degree of recovery from "osteoporosis" in young rats. Old rats are more refractory to changes in diet calcium, probably due to a smaller exchangeable calcium pool in the bone of older animals. Nevertheless, increasing the repletion diet calcium level from 0.22 to 0.78% increases bone density in old rats. This finding gives hope that, despite the refractoriness of mature bone, a diligent program of calcium supplementation might overcome

the effects of "osteoporosis" in older individuals. This study also shows that serum hydroxyproline may be a useful tool for the early diagnosis of "osteoporosis" in the aged when other clinical signs are still negative.

With increased diet calcium, femur potassium percent in ash is decreased in both young and old rats. Otherwise, bone (as a tissue) is chemically unaffected by diet treatment. Aging, on the other hand, creates some significant differences in femur mineral composition, including higher femur calcium, magnesium, sodium, copper, iron and zinc and lower femur potassium and manganese percent in ash.

Increasing diet protein results in increased bone turnover at both bone surfaces, as well as increased cortical area and bone density in young rats. Total cortical thickness, percent cortical area and cortical index, however, are reduced. Therefore, in the young growing rat, maximal skeletal growth rate stimulated by high diet protein may be incompatible with optimal skeletal characteristics. Excess diet protein results in osteopenia in old rats. This undesirable skeletal characteristic is not detectable by radiogrammetry or mineral analyses. High acid diets cause "osteoporosis" in rats, through increased osteocytic resorption of bone. However, chemically, the remaining bone (as a tissue) is unchanged by diet acidity.

The effects of graded levels of diet calcium, protein and acidity on soft tissue calcification were also studied in female rats fed diets with varying magnesium levels which

met National Research Council requirement, but which induced elevated kidney calcium levels as reported by some researchers. Low magnesium diets in the young growing rat induced nephrocalcinosis which was shown by histopathologic examination to be an intracellular-initiated dystrophic calcification. In the young rat fed low diet magnesium, increased diet calcium apparently limits nephrocalcinosis, although the effects of diet acidity are less clear. Nephrocalcinosis occurs as a further complication of metabolic acidosis following low diet magnesium and calcium deficiency. However, both chronic metabolic acidosis and alkalosis in the presence of low diet magnesium diminish the severity of nephrocalcinosis. Increased diet protein per se has no significant effect on soft tissue calcification.

Cholesterolemia was also observed to be affected by diet treatment. When the diet calcium to phosphorus ratio is either high or low, the serum cholesterol levels of both young and old rats are lower than when the diet calcium to phosphorus ratio is nearly equal. Excess diet protein results in decreased serum cholesterol levels in both young and old rats while diet acidity has a quadratic effect. Aging results in higher cholesterol levels.

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THE EFFECTS OF DIET CALCIUM, PROTEIN AND ACIDITY ON CALCIUM RETENTION IN THE RAT, PARTICULARLY AS RELATED TO THE INNER AND OUTER SURFACES OF TUBULAR BONE: A POSSIBLE MODEL FOR THE TREATMENT OR PREVENTION OF OSTEOPOROSIS

BY JOSEPH E. MILLIGAN

A thesis submitted to
The Graduate School
of
Rutgers, The State University of New Jersey
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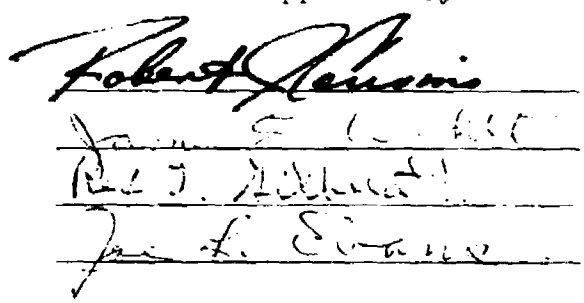
for the degree of
Doctor of Philosophy
Graduate Program in Nutrition

Written under the direction of
Professor Joseph Liston Evans
and approved by



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To Mary Ann

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I. INTRODUCTION

Metabolic bone diseases can be classified into two categories: osteomegaly (too much bone), and osteopenia (too little bone).

Osteomegaly results from osteopetrosis which is too little resorption of bone. It is characterized by nutritional secondary hypercalcitoninism (171,172,174,175).

Osteopenia can result from either too much resorption of bone or too little formation of bone. Too much resorption of bone (generalized osteodystrophia fibrosa) is characterized by either primary hyperparathyroidism, renal secondary hyperparathyroidism, or nutritional secondary hyperparathyroidism (166).

Too little formation of bone can be categorized into either too little mineralization of osteoid or too little formation of matrix. When osteoid is under mineralized, the young develop rickets and adults, osteomalacia (166).

Too little formation of matrix is osteoporosis (166). The term osteoporosis is sometimes used interchangeably with osteopenia to indicate an absolute loss of bone substance.

Osteoporosis, or more correctly osteopenia, is seen clinically in association with many conditions. It may be found in the presence of overt endocrinopathies such as hyperparathryoidism, hyperthyroidism, hyperadrenocoriticism, or acromegaly. It may be associated with malignant lesions (either primary or secondary) of the skeletal system. It may

also be found in association with disorders of the skeleton, e.g., osteomyelitis, disorders of the joints, e.g., rheumatoid arthritis, and in conditions of immobilization, either of a specific portion, or the entire, body (209).

A dietary cause of osteopenia has been shown in the horse (154), cow (316), pig (58), dog (137,170,268), cat (168,170,268,275), rat (88), mouse (278) and rabbit (153), and suggested in man (159,260). Diets which have inadequate Ca or excessive P can produce osteopenia. There is also recent evidence that dietary acidity can lead to osteopenia in the rat (231).

In that animal closest to man, the chimpanzee, the recommended Ca:P ratio is 1:0.77 (229). A 1955 USDA survey indicated that the average American consumed a diet having a Ca level barely adequate according to NRC recommendations (228) and a Ca:P ratio of 1:1.5 (298). A similar USDA survey in 1965 (299) showed the American diet to have a 1:1.6 Ca:P ratio. A 1975 USDA survey (299) showed the American diet to have a 1:1.7 Ca:P ratio. The 1975 USDA survey also showed that the production of milk and milk products (the source of 75% of our dietary Ca) had decreased by 15% during 1955-1975. During the same period, the production of meats (our richest P source) increased 16% (299). The USDA surveys concern gross food production. Food waste and uneven distribution are not considered. According to these USDA figures, the Ca available in the average American's diet does not meet the requirements for adults, and far less for the growing

population, pregnant or lactating women, and other individuals under Ca stress such as the military pilot in a high G environment or the astronaut in prolonged space travel.

These USDA surveys do not consider the so-called "junk foods" so prevalent in the American diet. These "junk foods", practically devoid of Ca and high in P, increase the imbalance in Ca:P ratio. Many of these foods, particularly soda pops, are also highly acidic and may therefore be a predisposing factor of generalized osteopenia. Since the publishing of these USDA reports, the quantity of meat protein (an acid-producing substance) consumed by the average American has further increased (300). Our changing food habits have the potential of making a bad situation worse.

The clinical diagnosis of osteopenia is made on the basis of symptoms of backache and pain associated with objective evidence of fractures and loss of skeletal mineral (200). However, 30% or more of the bone mineral can be lost before osteopenia is clinically detectable by radiography, even in patients with biopsy-proven hyperparathyroidism (248). Serum Ca levels are also not clinically diagnostic of osteopenia since 25-30% of the total body Ca can be lost with no change in serum Ca (159). The diagnosis of osteopenia, in practice, therefore implies a considerable loss of total mineral from the affected skeletal areas.

Clinically undetectable, generalized, chronic osteopenia predisposes the geriatric to injury (200). Osteopenia of the spine predisposes to pain and injury during bail out,

crash landing, or high G's experienced in modern military aircraft or during re-entry from space. It also complicates Ca loss during weightlessness (253). In a survey of bailout injuries, it was found that musculoskeletal injuries are exceedingly common and often leave permanent injury (157,233). Osteopenia may predispose to spinal injury which continues to be a major problem associated with sports injuries, and commercial automobile and aircraft crashes. Osteopenia may also predispose to spinal injury encountered among parachutists and recently in an increasing number of snowmobile accidents (157).

Generalized osteopenia has been found in persons with generalized malnutrition, such as were seen during World War II in concentration camps (200). Prisoners of war returned from Southeast Asia had peridontal disease (an early indication of generalized osteopenia) and it is well documented that the oriental diet on which these prisoners subsisted was seriously deficient in Ca (203).

It is therefore important to develop a diet which will prevent, limit or even reverse osteopenia. Since the foods high in Ca (such as milk and cheese) are also high in P, they cannot adequately be used to correct the Ca:P imbalance of the diet. A Ca supplementation of the diet may therefore be indicated as a means of eliminating or limiting osteopenia. Based on the possible relationship between dietary acidity and osteopenia, and an increase of acidic and acid-producing foods in the American diet, either reduction of the amount of

these acid and acid-producing foods in the diet, or the buffering of these foods may also be indicated.

Although the trials reported in this manuscript were directed toward the goal of limiting or reversing osteopenia, it was also expected that information would be obtained in these other medically important areas: (1) humans taking Ca supplements over a long period of time have shown a decrease in blood pressure and a lowering of serum cholesterol (173) and (2) rats on prolonged acid feeding have shown decreased serum Ca levels (231), a factor which could lead to hyperparathyroidism and its consequences such as nephrocalcinosis (71), renal lithiasis (236), and soft tissue calcification (153).

II. REVIEW OF LITERATURE

A. Bone and Bones

1. Definitions

"Although created and constantly molded by man, language and linguistic terms influence man's thinking to an astonishing degree. All too often, linguistic ambiguities are the cause of unnecessary confusion. It is then that a clarification of terms and their use is imperative."

"Dry bones consist of bone only; hence the use of the same word bone for the tissue bone and for the organ bone as a unit of the skeleton, which has confused and complicated the understanding of this chapter in biology. The pathologist speaks of bone tumors and means tumors of the bones. The anatomist speaks of membranous and endochondral bone formation instead of formation of bones. This leads, in the first case, to the greatest difficulties in establishing a natural classification of the tumors of the skeleton - the bones - and, in the second, to a perpetuation of the idea of two different types or kinds of bone tissue. There is only one type of development of bone. But there are two types of development of bones."

"There is a simple way out of these difficulties leading to great clarity:

Bone is a Tissue.

Bones are Organs."[1]

2. Bone as a Tissue

Bone is a tissue which is renewed by continuous anabolic (apposition) and catabolic (resorption) processes.

a. Bone apposition

Bone formation involves two distinct fundamental processes: (1) construction of an organic matrix and (2)

---

[1] Quoted from the textbook "Bone and Bones" by Weinmann and Sicher, Mosby, St. Louis, 1955.

deposition of bone salt in this matrix (171). Terms such as endochondral and intramembranous bone formation are misleading; they refer to growth of bones or organs rather than bone as a tissue. Bone is formed by osteoblasts only (246).

(1) construction of an organix matrix

In areas of developing bone, cells derived from primitive mesenchyme turn into osteoblasts, which appear to be responsible for laying down the intracellular organic matrix (osteoid). This has two main components: (a) the protein, collagen, arranged in bundles of long parallel fibers so that the bundles themselves run in many different directions, and (b) the so-called ground substance, consisting mainly of mucoprotein and mucopolysaccharide, resembling chondroitin sulfate, but not yet definitely characterized. The collagen fibers are embedded in this mucopolysaccharide ground substance, which may be regarded as a sort of cementing material (184). It has been shown that bone crystal morphology, as well as orientation, is in some way connected to the collagen fraction (246).

Osteoblasts lay down the organic matrix (the osteoid) on such surfaces as the chondroid core of the primary spongiosa, an already existing bone surface, and the inner layer of the periosteum. The Golgi apparatus in an osteoblast specializes in synthesizing and secreting the mucopolysaccharide cementing material while the endoplasmic reticulum of the osteoblast makes and secretes collagen. In time, relatively large amounts of the cementing substance

accumulate around each individual osteoblast and numerous bundles of collagen fibers come to be embedded in it (246).

Collagen is a protein, which has a unique amino acid content. Glycine residues make up approximately 33% of the molecule, proline and hydroxyproline residues each compose approximately 10% (184), with lesser amounts of other amino acids found, including hydroxylysine residues which are about 1% of the molecule (255). Collagen is made of units of molecular weight 300,000, which are known as tropocollagen. These are rodlike and have a length of 300 nanometers and a width of 1.4 nanometers. Each of these units consists of three peptide chains wound around each other in a right-handed helix, which is stabilized by hydrogen bonding between the chains (184).

To form collagen, these rods are laid down in a manner so as to first give rise to immature soluble collagen, which lacks covalent cross-links between tropocollagen rods and is maintained as a discrete pool. As the overall size increases, eventually the mature insoluble collagen is produced with extensive covalent cross-links between rods. Mature insoluble collagen forms cartilage and bone osteoid when combined with the appropriate mucoproteins and mucopolysaccharides (184). The synthesis of the hydroxyamino acids, hydroxyproline and hydroxylysine, is unique in that these two amino acids do not normally exist free in the fibroblast or osteoblast. Their precursors, proline and lysine, are first incorporated into a large intracellular peptide molecule,

protocollagen. Upon reaching an appropriate length, protocollagen then serves as a specific substrate for two hydroxylating enzymes which catalyze the hydroxylation of specific proline and lysine residues (255). Ascorbic acid is a cofactor for the hydroxylase (184).

Together the collagen fraction and the amorphous fraction comprise the organic matrix of bone (12,48). The amorphous fraction of bone supplies the internal environment for the crystals of bone mineral, for the collagen fibers, and for the cells incorporated within bone (osteocytes) (217).

(2) deposition of bone salt in the osteoid

The osteoblasts become trapped in the osteoid (organic matrix) which they manufacture and are, from then on, called osteocytes. Osteocytes are the living elements of bone. Their function is concerned chiefly with maintenance of the integrity of the organic matrix of bone. The metabolic activity of the osteocytes is reflected in the composition and chemical reactions of the amorphous fraction. The osteocytes may influence both the metabolism and the composition of the intercellular substance (196).

Under normal conditions, the osteoid is mineralized to a great extent (about 70%) almost immediately. The remaining mineralization is a slower, more gradual process (184). Bone mineral has been shown to consist of three Ca-P pools: a noncrystalline (amorphous) phase, an octacalcium phosphate phase, and a crystalline (apatite) phase (115). Forty percent of the total mineral in mature compact bone is present

in the form of the noncrystalline component. This percentage is even higher in younger bone (284,291). It has been postulated that in bone formation the cells, by some active process, form noncrystalline Ca-P and subsequently, part of this pool is stabilized while part is transformed, by dissolution and reprecipitation, into the crystalline constituent (85). It has also been suggested that crystalline hydroxyapatite is formed by a sequence of reactions beginning with Ca and phosphate ions in solution, forming in sequence amorphous Ca-P, octacalcium phosphate (with a Ca:P molar ratio of 8:6), and finally hydroxyapatite (115).

The apatites of bone are primarily hydroxyapatite which has the composition $Ca_{10}(PO_4)_6(OH)_2$ (230). Most of the apatites form very small or poorly formed crystals. Within the crystal lattice of bone apatite, ions of similar size are to a variable degree interchangeable. For example, F can exchange for hydroxyl or Sr for Ca. Also, the enormous surface area exposed by the small crystals permits extensive adsorption of materials onto the surface of the crystals (134). Bone also contains considerable water (265). Much of this water comprises a fluid that simply fills the open spaces in the bone matrix and presumably has a composition similar to that of the extracellular fluid. The remainder is tightly bound to the crystals, the so-called "hydration shell." Apparently, only certain ions can enter the hydration shell (230). Thus bone always contains a large variety of materials other than those which compose hydroxyapatite.

The amounts of some of these constituents which are not considered an integral part of the bone mineral are quantitatively important. It has been estimated that as much as 60% of the total body Mg, 25% of the Na, 90% of the carbonate, and 90% of the citrate may occur in bone (265), depending in part upon the nature of the diet. Bone formed with a normal Mg content, for example, may provide considerable reserve in time of need, whereas if the bone were formed with limited Mg, much less would be available (190).

The exact biochemical processes leading to calcification of the osteoid are not well understood. The concentration of Ca and P in the extracellular fluid is supersaturated in relation to hydroxyapatite. Crystal formation is initiated and the concentration of the extracellular Ca and P ions is sufficient to sustain crystal growth (184).

b. Bone resorption

Resorption of bone means the simultaneous removal of the bone matrix and minerals. It has been suggested that the three types of cells that characterize bone (osteoblasts, osteocytes and osteoclasts) are closely interrelated and are readily transformed one into the other, in both structure and function (136). As previously mentioned, bone is formed by osteoblasts only (246). Bone resorption, however, occurs by two different mechanisms: osteoclasia and osteolysis (32).

(1) osteoclasia

Osteoclasia defines a surface resorption of

bone tissue brought about by the action of osteoclasts. These cells are multinucleated giant cells, typically located in a Howship's lacuna during the active phase. Osteoclasia may occur on surfaces of trabeculae, on the subperiosteal and medullary surfaces, and on the surface of Haversian and Volksmann's canals (281). The exact nature of the mode of action of the osteoclasts is not known. It has been suggested that the solution of mineral in the osteoclastic resorption of bone is accomplished by chelation (216). Studies have shown that the osteoclastic brush borders are of particular significance in this function (177). During resorption, the osteoclasts manufacture acid phosphatase which lowers the pH in the cytoplasm and in the surrounding bone tissue.

(2) osteolysis

Osteocytic osteolysis defines a deep seated resorption centered around the activity of old osteocytes. In bone trabeculae, the most superficial, recently trapped osteocytes are relatively large and, like the osteoblasts, they contain alkaline phosphatase. At somewhat greater distance from the apposition surface, they are small, elongated and show no alkaline phosphatase. In the deepest part of the trabeculae, the osteolytic process is characterized by a number of changes: the osteocyte and its lacuna are larger; alkaline phosphatase reappears in the cytoplasm of the osteocytes and in the adjacent bone matrix; there is loss of mineral around the resorbing osteocyte; and there

is degradation of the polysaccharides in the matrix, degradation of the collagen fraction of the matrix and loss of matrix around the resorbing osteocyte (31).

(3) relative importance of osteoclasia and osteolysis

Numerous studies have shown that osteolysis is by far the most important mechanism in bone resorption (31,32,137), both under physiological conditions and in pathologically enhanced resorption. Osteoclasia is concerned with removal of already altered bone, e.g., necrotic bone at a fracture site or bone altered by osteolysis (32).

Bone renewal is a physiological process in which the resorptive phase aims at maintenance of proper plasma Ca levels. The natural target for resorption is the bone tissue richest in minerals, i.e., the oldest bone. The oldest bone is contained deep in trabeculae in the periphery of osteons. These are exactly the areas where osteolysis occurs (31).

Osteoclasia is a surface resorption. If osteoclasia were of any importance in the normal turnover of bone, this would, it seems, be a mismanagement of natural resources. Osteoclasia can concern only resorption of superficial, relatively Ca-poor bone and not the deep-seated, relatively Ca-rich bone (31,32).

(4) bone flow

The bone flow concept defines a view that bone tissue, and its variants dentin and cementum, are in

a constant flow from the site of apposition to the site of osteolysis. The concept that hard tissue is capable of movement in space was introduced already in 1691 by Havers but has been denied vigorously for about 275 years (169). Recent advances in the understanding of bone metabolism have reinstated this concept. Belanger and Migicovsky (30) showed that tritiated thymidine injected into young chickens could be traced in the nuclei of osteoblasts within two hours. After one day, radiothymidine was located in superficial osteocytes and after two days deeper in the bone tissue. After four days, the isotope began to disappear in the center of trabeculae and after seven days it was all gone. Belanger interpreted this as an indication that "the older osteocytes have already died and that a constant replacement stream is moving in from the border."

Ostocytic osteolysis and bone flow are interdependent events and can be summarized as follows: cancellous bone is formed on the surface of trabeculae, flows toward the deepest portion and is resorbed by osteolysis; compact bone is formed on the surface of the Haversian canal and flows peripherally to be resorbed by osteolysis in the peripheral lamellae; dentin is formed on the surface facing the pulp cavity and flows peripherally, the mode of a presumed dentinolysis being unknown; cementum is formed on the surface, flows toward the cementodentinal junction and is resorbed by cementolysis (169).

(5) hormonal control of bone resorption

Bone resorption aims at maintenance of proper plasma Ca levels and the rate is controlled by the plasma Ca through two hormones: parathormone (PTH) and calcitonin (CT).

Parathormone plays the principal role in Ca homeostasis (51). Its secretion is stimulated by hypocalcemia. One of the functions of PTH is to increase bone resorption and increase the transport of Ca from the bone to its surrounding fluids (160,262). The direct action of PTH on bone was first demonstrated in 1948 when resorption was observed adjacent to parathyroid glands transplanted to the surface of bone (18). The mechanism appears to have two stages. In the initial stage, PTH interacts with receptors found on the cell membrane. This interaction leads to activation of adenyl cyclase and increased conversion of ATP to cAMP (14). Careful measurements in vivo immediately after PTH infusion indicate that there is an initial short period of hypocalcemia. This is due to an initial increased influx of Ca into osteocytes, where Ca and cAMP act as messengers in preparing the cells for bone resorption (255,264). This osteocytic Ca mobilization occurs rapidly and does not depend on new RNA synthesis. The second stage involves a more prolonged mobilization of Ca. This is brought about by an increase in the number and activity of osteoclasts as a result of increased RNA, DNA and protein synthesis (42,235,288). An increased lactate production by rat calvaria has been reported 2 minutes after PTH administration (135) and a cell membrane depolarization

15 minutes later (218). The final result is an increase in efflux of Ca from bone cells due to the PTH stimulated osteocytic osteolysis (147). It has also been shown that within 30 minutes of PTH infusion, both the number and the density of intramitochondrial Ca granules in all bone cells increase, a finding which may be related to their transport activity (209,211,212).

Other functions of PTH are: (1) to decrease renal tubular P reabsorption and increase renal P excretion, which reduces plasma P and results in a reduced plasma Ca/P product (allowing increased plasma Ca by increased renal tubular Ca reabsorption and decreased renal Ca excretion); (2) to increase the solubility of Ca^{++} and HPO_4^{--} in plasma; (3) to increase intestinal absorption of Ca; and (4) to retard mineralization of osteoid (17,213,225,237).

Calcitonin is manufactured by the thyroid parafollicular cells of neural crest origin (186,240). Secretion of CT is stimulated by hypercalcemia (51). The function of CT is to decrease plasma Ca by inhibiting bone resorption (6,151,220, 234,263) and decreasing the transport of Ca from bone to its surrounding fluids (56,223,251,304). As with PTH, the effects of CT on skeletal tissue are mediated through cAMP. Calcitonin has been shown to activate adenyl cyclase and increase the conversion of ATP to cAMP in fetal rat calvaria incubated in vitro (34). The effects of CT and PTH on adenyl cyclase from fetal calvaria have been shown to be additive, indicating that the two hormones activate two separate enzyme

systems (132). Calcitonin has also been shown to stimulate the conversion of ATP to cAMP in rat bone in vivo (226). Calcitonin stimulates the production of adenyl cyclase in both osteoblasts and osteocytes (280). Calcitonin increases Ca influx but appears to depress Ca efflux in bone cells (124). It also dramatically increases the number and density of intramitochondrial Ca granules in osteocytes and osteoblasts (211,212). In addition, CT has been shown to increase the membrane potential of osteoclasts (218), to markedly reduce the ruffled borders of osteoclasts where osteoclastic resorption is assumed to occur (155,317), and to produce flattening of the ruffled border and loss of cytoplasmic coating from the cell membrane of osteoclasts (155). These actions of CT result in a direct inhibition of bone resorption through osteoclasia (104) and osteocytic osteolysis (174).

Other hormones have been shown to play a role in bone resorption and apposition. Growth hormone (72), estrogen (59,254), and progesterone in synergism with estrogen (290) have all been shown to oppose bone resorption. Prostaglandin, on the other hand, is a potent resorption - stimulating factor (294), while thyroxine apparently increases both resorption and apposition of bone (165,221).

3. Bones as Organs

a. Growth of bones

Multiple studies employing radiographic and other techniques have demonstrated the course of bone gain,

the phases of bone loss, and the principles of bone remodeling (89,108). Growth of long bones is a complex procedure. The two ends elongate at different and changing rates. A three phase simultaneous mechanism is involved in growth and elongation of long bones. The bone as a whole elongates by growth at the epiphyseal plate and the articular cartilage (46). Meanwhile, surface remodeling straightens the diverging walls of the conical region of the shaft. Subperiosteal apposition and endosteal resorption occur in the cylindrical portion of the shaft while subperiosteal resorption and endosteal apposition take place in the conical area (46,89, 185).

Growth of other bones is similar. Vertebrae grow from the epiphyseal plates and from the cartilaginous end plates facing the discs. Some mammals, e.g. man, rat and mouse, have no vertebral epiphyseal plates and their vertebrae grow from the cartilaginous end plates only. Flat bones grow from sutures (46).

b. <u>The epiphyseal plate</u>

In the growing individual there are three zones in the epiphyseal plate: (1) zone of resting cartilage (zone of proliferation); (2) zone of columnar cartilage (zone of maturation); and (3) zone of vesicular cartilage (zone of hypertrophy). The first zone is narrow and relatively poor in cells. The other two zones are of about equal thickness and the cells increase in size toward the metaphyseal end (46).

Proliferation of cells occurs by mitotic division of resting cartilage cells, a process controlled by growth hormone. The maturation and hypertrophy of cartilage is controlled by thyroxin (46).

The matrix between the most distal vesicular cartilage cells becomes mineralized. This is the zone of provisional calcification. The vesicular cells facing the metaphysis are large with relatively little chondroid matrix (condroid core). These cells are penetrated by vessel brushes from the metaphysis and disappear. Osteoblasts accompanying the vessel brushes lay down osteoid on the surface of the chondroid core. The core continues for some distance through the primary spongiosa and is completely resorbed in the secondary spongiosa (46). The resorbing cell is the osteocyte and, since chondroid matrix is the target for this resorption, the process is called chondrolysis. The chondroid matrix is resorbed in the center of the bony trabeculae where there are no surfaces. Therefore chondroclasia via osteoclasts is not a factor (31,32).

Cessation of longitudinal growth coincides with sexual maturity. The estrogens and testosterone counteract the growth hormone and proliferation of resting cartilage ceases. The existing columnar and vesicular cartilage continues to mature under the influence of thyroxin. The plate therefore gradually diminishes in thickness and finally disappears. Communication is established between the epiphysis and metaphysis. This process is called closure of the epiphyseal

plate (46).

c. The articular cartilage

In the growing individual, articular cartilage grows in two directions from a growth center at the junction between the middle and inner third of the cartilage. Growth toward the epiphysis mimmicks the growth of the epiphyseal plate but is much slower. Longitudinal growth from the articular cartilage contributes only 3% of the total length of a long bone. Growth toward the surface aims at replacement of surface cells lost by wear and tear (46).

Longitudinal growth from the articular cartilage also ceases with sexual maturity. The proliferation of cells ceases and the inner part of the cartilage is mineralized. This inner part is called the zone of calcified cartilage. Growth toward the surface, however, does not cease. The surface cells are continuously renewed (46).

d. The synchondrosis

A syncondrosis is a cartilaginous joint between two bones, e.g., the bones of the base of the skull. A synchondrosis looks like two epiphyseal plates with a common zone of resting cartilage. The plates cause growth in opposite directions. Growth and closure are the same as for the epiphyseal plates (46).

e. The suture

A suture between flat bones consists of a central portion of well collagenized fibrous tissue. Toward the periphery the suture is less dense and the cells gradually

become larger. Facing the bone is a single or double layer of osteoblasts which lay down bone tissue. The flat bones thus grow by expansion from the sutures (46).

B. Osteopenia

Osteopenia is simply too little bone. It can be a result of either too little formation (osteoporosis, rickets, or osteomalacia) or too much resorption (generalized osteodystrophia fibrosa).

1. Osteoporosis

Osteoporosis is defined as a generalized metabolic bone disease characterized by osteopenia due to too little formation of matrix (184). The term osteoporosis is often erroneously used as a synonym for osteopenia.

a. Etiology

Causes of osteoporosis are not fully understood. The disorder occurs in senility, in disuse atrophy, in a variety of obscure hormonal imbalances (involving the adrenals, thyroid, or pituitary), and in some other conditions. Causative mechanisms are thought to include absence of the stimulation coming from the stresses and strains of movement, malnutrition (especially with respect to proteins), and possibly deficiency of estrogen (human post-menopausal), excessive adrenal cortical hormone, hyperpituitarism, and hyperthyroidism. Vitamin C deficiency and copper deficiency also result in osteoporosis. Considerable attention is presently being directed toward Ca deficiency as a cause of osteoporosis (281).

b. Pathologic anatomy

In osteoporosis there is, by definition, too little bone. Osteoblasts are few and far apart along the apposition surfaces. The remaining ones are atrophic. Bone trabeculae in the metaphysis are few and slender. Cortical bone is thinner than normal and the Haversian canals are wider. Bone resorption goes on at a reduced rate, although resorption exceeds production. The remaining bone is apparently normal (281).

2. Rickets and Osteomalacia

Rickets and its adult counterpart, osteomalacia (softening of bones), are defined as generalized metabolic bone disease characterized by osteopenia resulting from too little mineralization of matrix (281).

a. Etiology

Rickets occurs when the product of Ca^{++} and HPO_4^{--} in blood plasma decreases too far to allow mineralization of osteoid. If the product is lowered mainly because of inadequate Ca^{++}, it is referred to as low Ca rickets, and if due to insufficient HPO_4^{--}, it is a low P rickets. Anything that lowers this product is thus of etiologic importance. Vitamin D deficiency is one of the many causes, but while vitamin D deficiency equates rickets, rickets does not equate vitamin D deficiency. Other causes of osteomalacia and rickets include dietary Ca and P deficiency, chronic gastrointestinal disorders which cause interference with mineral absorption, and formation of insoluble salts

in the intestine (281).

b. Pathologic anatomy

The classic macroscopic feature of rickets and osteomalacia is the "rosary border" of the costochrondral junctions. The ribs break like cardboard without a snap. Deformities and compression fractures of vertebrae are lesions of more advanced cases and fractures of long bones may also occur.

The diagnostic features are found in the epiphyseal plates, costochrondral junctions, and in adjoining trabeculae. Mineralization does not occur in the zone of provisional calcification. The distal row of vesicular cartilage cells is not penetrated by vessels from the metaphysis. The vessels continue up into the cartilage matrix. Unopened, large cartilage cells grow down into the metaphysis, either in single rows, or in large conglomerates. The epiphyseal-metaphyseal line is therefore very irregular. There is usually tremendous osteoblastic activity along the trabeculae with a wide seam of non-mineralized osteoid which stains pink with hematoxylin and eosin in a histopathologic section. The excessive osteoid accounts for the swelling of the epiphyseal-metaphyseal area (281).

In osteomalacia, the diagnosis rests upon the demonstration of the osteoid seams only, since the epiphyseal plates are closed in the adult (281).

3. Osteodystrophia Fibrosa

Generalized osteodystrophia fibrosa is defined as

a generalized metabolic bone disease characterized by osteopenia resulting from too much resorption (281).

a. Etiology

A marked increase of bone resorption is a constant feature of fibrous osteodystrophy. It results from a prolonged and excessive stimulation of bone by PTH. This may result from primary hyperparathyroidism which is rare in animals, or secondary hyperparathyroidism in response to hypocalcemia. Secondary hyperparathyroidism is frequent in animals with chronic renal disease or chronic nutritional imbalance, such as vitamin D deficiency, Ca deficiency, excess dietary P (281), or possibly excess dietary protein (47,150,301) and/or acidity (231,261).

b. Pathologic anatomy

The most important feature of generalized osteodystrophia fibrosa is excessive resorption (281). Osteolysis is the earliest and most important mode of resorption and osteoclasia is of late occurrence, concerned with removal of bone already altered by osteolysis (31,32).

The bone loss is generalized but there are sites of predilection where the lesions appear earlier and reach more severe degree with time. The hierarchy of the bone loss is, in decreasing order: the jaw bones (especially the alveolar bone), other skull bones, ribs, vertebrae, and long bones. The selective involvement shows that cancellous bone is more predisposed to excessive resorption. When the resorptive phase is accentuated in hyperparathyroidism, the bone with

the greatest basal rate naturally suffers most (281).

Resorbed bone is replaced by fibrous tissue. The degree of proliferation of fibrous tissue varies greatly. Depending on the degree of fibrosis, volume changes of the bones (as organs) may occur. We recognize either decreased volume, unchanged volume, or increased volume of bones with osteodystrophia fibrosa (184,281).

Increased osteoblastic activity is seen frequently. It represents an attempt to replace the lost bone. The newly formed bone matrix is poorly mineralized and these osteoid seams reflect one of the functions of PTH, i.e., to retard mineralization of osteoid. If minerals gained by resorption were immediately deposited in newly formed osteoid, they would not be available for compensation of hypocalcemia (281).

C. Dietary Models for the Treatment of Osteopenia ("Osteoporosis")

Despite the complex mechanisms of growth of long bones, with apposition and resorption at both the subperiosteal and endosteal surfaces, and continuous remodeling beneath these surfaces even after epiphyseal closure, it is still possible to present a simple midshaft model of bone gain and loss, ignoring the complexities of growth and remodeling.

It has been radiographically shown that subperiosteal apposition occurs throughout life, with an adolescent spurt; and that endosteal surface change is characterized by preadolescent loss, adolescence through midadulthood gain, and late adulthood loss (108). The result is a large increase

in bone mass during childhood through adolescence, a small and gradual increase from adolescence through midadulthood, and a gradual and steady decrease in bone mass from mid-adulthood through the remainder of life.

A frequently asked question is: "why this apparent inevitable progressive 'osteoporosis' with age?" A number of hypotheses have been advanced to explain bone gain and loss, including activity effects, hormonal action, and nutritional factors. Even though these factors have been shown to aggravate the severity of "osteoporosis", none of these hypotheses have adequately explained the incidence of "osteoporosis" with age.

The lower incidence of "osteoporosis" among blacks is not due to greater activity, but rather has a genetic epidemiologic nature. Blacks have larger bones at all ages than do whites, and less bone is lost from larger, more compact bones than from smaller, less compact bones (108,301). Inactivity is of no consequence in bone loss, except in the case of total immobilization (108,261,301), or the prolonged effects of zero gravity during space flight (253).

Growth hormone is probably responsible, at least in part, for continuous subperiosteal apposition throughout life (108), but the suggested protective effect of estrogens against endosteal loss has not been substantiated (107,108, 199,261).

Adult bone loss has not been shown to be completely reversible by such nutritional factors as Ca feeding, vitamin

D, fluoride, and manipulation of the Ca:P ratio (106-108, 199,261,301). These facts do not preclude a continuing search for models that will enable us to slow or even reverse progressive endosteal bone loss, and to further enhance subperiosteal apposition. Calcium therapy is certainly one such model. Another model might be the function of bone mineral as a buffer base.

1. Calcium

The role of Ca deficiency in "osteoporosis" is yet to be defined. But "osteoporosis" has been experimentally produced in many species (58,88,137,153,154,159,168,170,260, 268,275,278,316) by Ca deficiency and/or P excess.

In a series of long term experiments on rats and mice (8,278), it has been found that aging in these species is associated with the development of "osteoporotic" changes in the skeleton which are analogous to those observed in man. Unfortunately, this trend could not be fully counteracted by increasing the dietary concentration of Ca. Many other experiments have also indicated an inability of Ca feeding to remove "osteoporosis" (106-108,199,261,301).

Despite these setbacks, a search for Ca supplementation as a model for reversing "osteoporosis" continues. It has been shown that Ca limits mineral loss from bone cells in tissue culture (250). Furthermore, alternating infusions of Ca and P increase bone mass by enhancing bone formation (245).

Recent evidence suggests that Ca supplementation reverses "osteoporosis" in rats (111). It has also been

suggested that oral Ca supplements may promote skeletal remineralization in humans, based on studies of patients with renal osteodystrophy (64). Calcium supplementation has been somewhat effective in improving breaking strength of vertebrae in rats with disuse "osteoporosis" (267). Long term Ca repletion of previously Ca depleted dogs has been effective in reversing "osteoporosis", and it has been suggested that this same therapy may be effective in humans (170). Calcium therapy has also been shown to be effective in treating human peridontal disease which is an early form of generalized "osteoporosis". This indicates that long term Ca therapy may be effective in treating or preventing "osteoporosis" of long bones which is a chronic manifestation of Ca deficient or P excess diets (173,176).

2. Dietary Protein, Acidity and Alkalinity

Qualitatively, bone is a very minor buffer when compared to the bicarbonate and hemoglobin buffer systems, but quantitatively the buffering capacity of the basic salts of bone is a sizeable one considering the proportion of bone in the body (309). Clinical "osteoporosis", or more correctly, osteopenia, is believed to be due to increased bone resorption and may be corrected by any mechanisms which decrease bone resorption (199). A slight Ca loss over an extended period, such as due to buffering of acid, could cause "osteoporosis" (261). It has long been known that bone responds to an acid load by dissolution of its basic salts (4,13). Bone acts to buffer hydrogen ions and in so doing

releases Ca ions (282). Bone Na, Mg and carbonate can contribute to the regulation of acid-base balance, and if the bone crystals are sacrificed, phosphate is made available to neutralize hydrogen ions (134). It has therefore been postulated that "osteoporosis" may be due to life long utilization of buffering capacity of the basic salts of bone in response to an acid ash diet (301). Nutritional studies in this respect are therefore important. It is well established that net acid production is related to nutrition. The herbivorous rabbit for example excretes an alkaline urine, while the carnivorous dog excretes an acid urine (77). The urine of omnivorous man is acidic, while that of vegetarian man is alkaline (301).

Increased diet protein (acid ash) has been shown to stimulate bone resorption (78), while acid stress lowers serum Ca in rats (231). Excessive administration of NH_4Cl to normal adult male rats was shown to cause the development of "osteoporosis". The "osteoporosis" was due to loss of bone matrix and bone mineral associated with increased bone resorption (19). Ammonium chloride induced "osteoporosis" has similarly been shown in oophorectomized female rats (21). Other studies have also shown that chronic metabolic acidosis decreases bone carbonate content and increases bone resorption in rats (22) and dogs (61).

Protein, acidity and alkalinity might affect Ca retention through a number of mechanisms: excretion in the urine and feces, absorption through the gut, direct action

on bone, or hormonal action.

In normal subjects urinary Ca is related to dietary Ca (163). Excretion of Ca that is excessive in relation to intake may be due to an increased filtered load of Ca, decreased tubular reabsorption, or a combination of the two (193,232). It has been shown that an excessive excretion of urinary Ca can be produced in man by metabolic acidosis caused by feeding an acid producing (high protein) diet or through NH_4Cl ingestion. Inorganic acid feeding also causes an increase in urinary Ca excretion in man (47,193,287), as well as in pigs (182,183), and other animals (96,97). Furthermore, increasing protein in the diet, while maintaining a constant acidity, can in itself cause a slight but not necessarily abnormal increase in urinary Ca (163). Conversely, while urinary Ca in normal man is not materially affected by ingestion of NaH_2PO_4 (93), it has been shown that alkali administration (238) and sodium bicarbonate feeding (86) reduce urinary Ca in human patients with hypercalciuria. Therefore, in general it can be stated that urinary Ca is elevated by metabolic acidosis and reduced by metabolic alkalosis. This is probably due to a direct action of extracellular fluid pH on bone mineral, producing small undetectable changes in plasma Ca and the renal filtration load of Ca (202). These changes are probably masked, however, by the effects of acidosis in reducing and alkalosis in increasing the protein binding of Ca, since it is presumed that acidification of the renal tubular fluid in man

enhances reabsorption of Ca complexes by enhancing their dissociation into ionized forms (232).

Metabolic acidosis has generally been shown to produce no change in fecal Ca (93), although one study shows increased fecal Ca with acidosis in man (197).

Numerous studies both *in vivo* (188) and *in vitro* (122, 187,189) have shown that amino acids increase the solubility of Ca salts. A study using the small intestine of the rat shows that Ca is transported across cells mainly, if not solely, in the ionized state (270), presumably due to acidification. Likewise, other studies (5,90) have shown that diets producing acidic conditions in the small intestine of rats increased Ca absorption. Increasing protein levels in the diet have also been shown to increase Ca absorption in both children (128,273) and human adults (178,215,243).

A major point to consider when reviewing the effects of protein and acidity on Ca excretion and absorption is net Ca balance. While the effects of protein and/or acid diets on Ca excretion and absorption are fairly well defined, the overall effects on Ca retention are less clear. Diets containing HCl have been shown to decrease Ca retention in rabbits (116) and growing children (289,318), while $NaHCO_3$ had the opposite effect (289,318). Diets containing NH_4Cl which is metabolizable to acid decreased Ca retention in children (244) and adults (192).

Protein (acid ash) diets decreased Ca retention in man (150,231). Studies with growing rats (5,90), on the other

hand, have shown that acidic diets increase Ca retention, while several studies with adult humans have shown protein (acid ash) diets to likewise increase Ca retention (178,215, 243). Still other studies have shown high protein diets to have no effect on Ca retention in children (128,273).

The mechanisms through which metabolic acidosis induces negative Ca balance in some studies are not clear. Barzel (20) postulated that lowering of pH per se directly increases Ca mobilization from bone. On the other hand, Wachman and Bernstein (302) proposed that metabolic acidosis augments Ca mobilization from bone by either increasing PTH secretion or augmenting the action of PTH on bone. The complex mechanisms involved in Ca balance may in part explain the apparently conflicting postulates (20,302). As has been seen, Ca balance is affected by urinary Ca excretion and gastrointestinal absorption as well as by Ca mobilization from bone. In the kidney, acidosis directly inhibits the tubular reabsorption of Ca, but augments the effect of PTH to increase tubular reabsorption of Ca (106). Furthermore, PTH (4), vitamin D (15,118), and CT (104) affect Ca mobilization from bone, and the same hormones affect urinary Ca excretion (307) and gastrointestinal Ca absorption (15,118). Metabolic acidosis may affect these multiple and interacting hormonal actions in multiple organs. Therefore, it is difficult to elucidate the mechanisms involved in negative Ca balance in metabolic acidosis.

It is obvious that much information is still needed on

the effects of protein, acidity, and alkalinity on Ca retention. A major criticism (163) of much of the work so far accomplished, is that few researchers paid any attention to dietary Ca and P levels and ratios, and dietary acid/base status. It should be noted that in those studies where Ca:P ratios (5,90,178,215,243) and acid/base balance (5,90, 178,243) were controlled, whole body Ca retention was improved, owing to the protein or acid effect of increasing Ca absorption.

It is therefore desirable to conduct controlled studies in which these variables are eliminated. Furthermore, when developing protein, acid, or alkaline diets as models for the possible treatment or prevention of "osteoporosis", particular emphasis should be placed on the effects of these parameters on bone and its active apposition and resorption sites.

D. Methods of Detecting Osteopenia ("Osteoporosis")

1. Radiographic Measurements

Radiographic measurements of total tubular bone width, medullary cavity width, cortical thickness, cortical area, percent cortical area and cortical index are more useful than the general terms "osteopenia" and "osteoporosis" when examining the effects of dietary factors on bone (110). Tubular bone width indicates the relative rates of subperiosteal apposition. Medullary cavity width indicates the relative and proportional endosteal loss/gain/loss of bone.

The cortical area and percent cortical area within the anatomical bone envelope are an indication of the mechanical properties and strength of bone (109,110). Cortical index also indicates bone strength.

Thus, the use of radiogrammetry provides information on changes at both bone surfaces not possible by other techniques (109). However, a problem in evaluating injuries is the limitation of diagnostic X-ray as an aid to finding small fractures (157). For instance, in studies concerning restraint of Rhesus monkeys in body casts, mineral loss is less than can be detected in standard radiographs (233). It has been suggested that 30-50% of bone mineral must be lost or gained before such changes are clearly apparent from radiographic image (68).

Nevertheless, in extreme cases the degree of cortical thinning frequently parallels that of cortical bone mineral density. Therefore, radiographic cortical thickness measurements usually are adequate for the diagnosis of pronounced "osteoporosis" (219). Furthermore, in rats it has radiographically been shown that "osteoporosis" exists following Ca depletion, even though Ca calculated as a percent of fat free dry bone remains unchanged with dietary treatment (111).

Regardless of the difficulties in diagnosing osteopenia with radiogrammetry, it is still useful to examine the correlation of radiographic analysis with other ancillary measurements such as gravimetry, mineral analyses, serum hydroxyproline determinations, and histologic examinations.

2. Gravimetric Measurements

It has been shown that bone Ca, when expressed as a percent of fat free dry bone, might be unchanged in "osteoporotic" conditions (111). The same therefore could be expected of bone Ca expressed as a percent of ash. Therefore, gravimetric measurements are more accurate indicators of osteopenia than the above measurements (167). The reasoning is as follows: as bone mineral decreases, bone weight also decreases, so bone ash per bone weight remains basically unchanged; however, as bone weight decreases, bone volume remains unchanged due to fibrous replacement; therefore ash per cubic centimeter (cc) of bone decreases with demineralization. It is obvious then that bone ash per cc should be an accurate indicator of osteopenia, which by definition is too little bone within the anatomical bone envelope (i.e., low bone density).

Specific gravity is another means of measuring bone density (19,170) and as such should correlate well with ash per cc (308). Changes in specific gravity (density) of bone can only be affected by a difference in ash, since volume remains unchanged (167).

3. Mineral Analyses

Despite the use of radiogrammetry and gravimetry as indicators of osteopenia, mineral analyses are not to be neglected. Absolute loss of skeletal Ca and ash has been repeatedly shown during "osteoporosis" (8,80,278). Trace mineral analyses may also be of value, especially when

concerned with the supposed function of some of these bone minerals in buffering metabolic acidosis caused by feeding acid ash diets (4,13,134,282).

4. Hydroxyproline Determinations

Relatively large amounts of hydroxyproline (HP) are found in collagen, but no other body constituent contains significant amounts of the amino acid (184,255). Hydroxyproline is synthesized by the hydroxylation of large polypeptides as one of the terminal steps in the formation of collagens, and apparently there is no other mechanism for synthesizing HP in vertebrates (255). These relationships make HP a convenient, naturally occurring label for studying the metabolism of collagen, and the presence of HP in tissues, plasma, or urine can be used as a measure of collagen or of degradative products of collagen (44,121,247).

Since a nondialyzable urinary HP peptide has been shown to be a reflection of collagen formation, a measure of both total HP excretion in the urine and the amount of this unique peptide may provide a distinction between, and a measure of destruction of mature collagen and of collagen synthesis (121,255).

As a rule, the levels of free and bound HP in the serum and urine have been shown to be a valuable index of bone matrix metabolism (44,161,310,311). The possible exceptions to this rule would be during certain pathological or physiological conditions such as: intravascular hemolysis in which erythrocyte prolidase would cause a sharp reduction in

urinary peptide HP (161); or the regression of liver cirrhosis and carrageening granuloma (310), which are involutionary examples of collagen resorption resulting in an increase in serum free HP; or postpartum involution of the uterus which would also result in an increase in serum free HP (310,311).

It is interesting to note that the involuting uterus has no effect on the serum bound or urinary free and bound HP levels (161,310).

5. Histologic Examination

The three important histologic features of bone indicative of remission of "osteoporosis" are: (1) cementing lines, (2) retained chondroid core, and (3) the appearance and persistence of excessive subperiosteal bone.

Belanger et al. showed that cementing lines merge from areas of diffuse matrix basophilla or metachomasia and that they, thus, represent arrested osteocytic osteolysis (29).

The extension of the secondary spongiosa (with retained chondroid core) toward the diaphysis is likewise an expression of retarded osteocytic osteolysis (169).

The appearance and persistence of excessive subperiosteal bone also represents too much formation and too little resorption of bone. The delay in remodeling of cortical bone is simply a result of delay in formation of resorption cavities which, in turn, is caused by retardation of osteocytic osteolysis (305).

E. Nephrocalcinosis and Cardiac Calcinosis

1. Factors Affecting Nephrocalcinosis

Nephrocalcinosis is a common entity. For example, it occurs secondary to ischemic necrosis of ovine kidneys (158) and has been observed in other species due to a variety of causes. One study reports calcified kidney lesions commonly occurring in 5% of all dog autopsies unassociated with any other calcium lesion or disturbance (45). Another report shows 39.5% of all dogs, regardless of age or breed, with calcified kidney lesions unassociated with clinical disease (70). Nephrocalcinosis has been induced experimentally in the rabbit by dietary P supplementation (153) and in mice, by increasing dietary Ca (278). In man, reduced urinary content of Mg (91), P infusion for treatment of hypercalcemia (57), and a lack of a specific peptide inhibiting calcification (140) have all been considered to be important causes of nephrocalcinosis. From the voluminous information on the etiology of nephrocalcinosis and nephrolithiasis in the rat, the following factors have emerged as of major importance:

1. systemic acidosis or alkalosis (120),
2. chronic systemic acidosis (119),
3. excess carbonates in conjunction with Mg deficiency (102),
4. reduced urinary content of Mg (283),
5. elevation of urinary pH with reduction in urinary Mg excretion (119),

6. dietary Mg deficiency (102,142,239),
7. Mg infusion (35),
8. Mg and Ca deficiency (258),
9. Mg and Ca deficiency with P excess (101,239),
10. P infusion (35),
11. high P diets (80),
12. low P diets (92),
13. high Ca diets (125),
14. excess dietary Ca and P (117),
15. P supplementation in vitamin D induced hypercalcemia (285),
16. high dietary cadmium (244),
17. prolonged intravenous infusion of pure parathormone (71),
18. water restriction (283),
19. vitamin B_6 deficiency (2,9,92), and
20. reduced citrate excretion in the urine (125).

It is apparent, therefore, that no factor can solely be held responsible for the development of nephrocalcinosis, either under experimental conditions or in clinical situations.

2. Effect of Diet Magnesium

Woodard (313) observed nephrocalcinosis in young growing female, but not male, rats fed semipurified diets which met NRC requirements (227), and determined that the macromineral mixture was the dietary component causing nephrocalcinosis. The responsible macromineral factor may have been

deficient Mg since Hurley et al. (142) showed low Mg (0.04%) diets fed to female rats cause a trend of high kidney Ca (even though this level meets NRC requirements). Martindale and Heaton (210) have made a similar observation. Such findings are indicative of Mg deficiency, even though previous work had shown 0.04% diet Mg to be a level that provided optimum growth (179) and normal tissue Mg concentrations (214). The faster growing animals are those which may be expected to exhibit more marked Mg deficiency symptoms (101).

Among the nutrient deficiencies which result in nephrocalcinosis and nephrolithiasis, the biochemical mechanism in Mg deficiency is one of the least understood. Feeding of a low Mg diet to young rats results in a typical syndrome which includes skin hyperemia and soft tissue calcification. The hyperemic state is believed to be a consequence of mast cell degranulation. The mechanism(s) for the accumulation of Ca in the kidney and other soft tissues (heart, aorta, muscle) is not clearly understood (60). However, the extensively documented changes in mineral concentrations in tissues (particularly renal tissue) of Mg-deficient animals (24,101,102,112,113,133,207,271,272,277,303,306) provides a convenient tool for studies of mechanisms leading to soft tissue calcification (146).

During Mg deficiency, chemical alterations in tissue are characterized by an increase in calcium concentration and a decrease in Mg concentration in the heart, and an

increase in Ca levels of the kidney. Bellavia et al. (35) showed that Mg also accumulates in renal tissue, probably due to codeposition with the Ca complexes.

3. Effect of Diet Calcium

Ingestion of diet Ca levels greater than NRC recommendations has been reported to result in calcification of soft tissues (297). One study has shown that increasing diet Ca from 0.32 - 0.64% and from 0.18 - 0.69% results in increased heart and kidney Ca with a concomittant decrease in heart and kidney Mg (266). By the same token, Ca depletion was shown to decrease heart and kidney Ca while causing a rise in heart and kidney Mg deposition (266). Another study (148) has also shown a decrease in heart Mg levels during myocardial Ca accumulation.

4. Effect of Diet Acid

Increasing the plasma concentration of H ions by counterbalancing diet Ca has been shown to protect against intracellular myocardial Ca accumulation (148).

5. Effect of Age

It has been shown that both heart and kidney Ca and Mg deposition increases with increasing age in the rat (266).

F. Cholesterolemia

The influence of various dietary factors on cholesterol metabolism in man and animals has received considerable attention by researchers during the last 20 years, however, the exact mechanism is still unclear. Several investigators

have shown that areas with soft drinking water have a higher mortality rate from all forms of cardiovascular disease (43, 274). Others have reported that the incidence of deaths due to ischemic heart disease is lower in areas with hard drinking water (10,11,74,164).

There is a close positive correlation between the hardness of drinking water and the Ca concentration of the water (73,84). This correlation has stimulated investigation of the relationship between dietary Ca and cholesterol metabolism in several species. It was shown in rabbits that increased diet Ca overcomes the cholesterolemia that occurs during acute starvation (144). In a further study in which rabbits were fed 0.02, 0.8 and 1.6% diet Ca, cholesterolemia occurred only in rabbits fed the Ca-deficient diet (143).

In rats, it has been reported that a decrease in plasma cholesterol occurs when feeding diets high in Ca (314). It has also been found that with increasing diet Ca (0.08 to 1.2%) blood lipid levels in rats decrease (99). Further long term studies in rats have shown a decrease in serum total lipids, phospholipids, cholesterol and triglycerides when diet Ca levels are increased from 0.08 to 2.0% (100).

In man, it has been shown that the incidence of cholesterolemia decreases when patients are given Ca supplements (63,315). Humans with peridontal disease being given Ca supplements over a long period of time have shown a decrease in serum cholesterol (173). It has also been shown that cholesterolemia is less severe when subjects are fed a

high saturated fat diet with Ca supplementation. However, the hypocholesterolemic effect of Ca is not evident in subjects fed polyunsaturated fat diets (40). Intravenous Ca supplementation is ineffective in reducing cholesterolemia (204).

It is now fairly well established that oral supplementation with Ca decreases the concentration of plasma cholesterol (3,41,63,315). Other dietary factors, however, have also been shown to have a hypocholesterolemic effect. A high Ca:P ratio has been shown to reduce the absorption of cholesterol in rats (117,293). High diet P in relation to Ca has also been shown to reduce cholesterol absorption in rats (293). High diet Mg is likewise effective in this respect (293). In addition, it has been suggested that a decreased diet Zn:Cu ratio is hypocholesterolemic (162).

Certain vitamins have also been shown to affect serum cholesterol levels. Vitamin D supplementation increased serum cholesterol in man (75), while high doses of vitamin D caused greater liver cholesterol in rats (127). Large amounts of nicotinic acid reduced serum cholesterol while pyridoxine deficiency caused a small rise in serum cholesterol in rats (127). High dietary vitamin A (83) and vitamin E (65) reduced serum cholesterol in rats. Folic acid feeding caused an increase in serum cholesterol in hypocholesterolemic patients with macrocytic anemia (25).

A relationship between diet protein and cholesterolemia has been shown by several investigators. The feeding of a

low protein diet results in an elevated plasma cholesterol level in the growing chick (194). Similar observations were reported in the Cebus monkey (208). The serum cholesterol level in another study, conversely, was significantly reduced when casein level in the diet was raised from 6 to 25% (127). Still another study suggested that raising the diet protein level from 25 or 30 to 46.8% was responsible for lowering serum cholesterol in rats (1).

III. MATERIALS AND METHODS

A. Experimental Design and Diet Composition

1. Trial 1

The experimental arrangement of treatments in Trial 1 was an incomplete factorial design consisting of 3 Ca levels (0.22, 0.48, 0.78%) and 3 protein levels (9, 18, 36%). For each Ca and protein level, 2 more variables, diet acidity (natural and acid added) and age (mature and young growing) were added in a 2 x 2 factorial design to give a total of 20 treatment groups with 10 repletion diets (Table 1). Four replications were allotted for each treatment. One hundred and four rats were fed a low Ca (0.16%) depletion diet for 7 weeks. Three rats from each age group were randomly selected from each replication and sacrificed as controls. The remaining rats were fed the 10 repletion diets for 6 weeks.

The basal diet (Tables 2, 3, 4 and 5) was supplemented with Ca lactate to give 167 mg calcium per 100 g of depletion diet, and with Ca carbonate to give 220, 480 and 780 mg Ca per 100 g of repletion diet, as shown in Table 6. Differences due to addition of Ca were corrected by replacing an equivalent amount of alphacel. The P content of all the repletion diets was maintained at 0.4% in order to keep the Ca:P ratio between 1:2 and 2:1 as suggested by Bethke et al. (39) and Hansard et al. (123). Protein levels of the repletion diets were achieved by varying the casein and D-L

methionine levels of the basal diet. Sucrose and starch replaced equivalent amounts of casein and D-L methionine (Table 7) to make all the diets equi-caloric. The acid-added diets were individually titrated to pH 5.2 with 2 N HCl as shown in Table 8. The percent dry matter of all the repletion diets was equilibrated during acid titration by the addition of deionized distilled water as shown in Table 8.

2. Trial 2

The experimental arrangement of treatments in Trial 2 was a 2 x 4 factorial design with 2 levels of Ca (0.48, 0.78%) and 4 levels of acidity (pH 5.0, 5.8, 6.6, 7.4). Two age groups (mature and young growing) were added as additional variables to give a total of 16 treatment groups with 8 test diets (Table 9). Four replications were alloted for each treatment.

Seventy-six rats were fed a control diet, the basal diet supplemented with Ca carbonate to give 480 mg Ca per 100 g of control diet, for 14 days (Table 10). At the end of this control period 6 rats from each age group were randomly selected and sacrificed as controls. The remaining rats were fed the 8 test diets for 7 weeks.

The basal diet (Tables 2, 3, 4 and 5) was supplemented with Ca carbonate to give 480 and 780 mg Ca per 100 g of test diets, as shown in Table 10. Differences due to addition of Ca were corrected by replacing an equivalent amount of alphacel. The P content of all the test diets

was maintained at 0.4% as in Trial 1. The pH levels of the test diets were attained by titration with 2 N HCl or 2 N NaOH as shown in Table 11. The percent dry matter of all the repletion diets was equilibrated during acid or alkali titration by the addition of deionized distilled water as shown in Table 11.

B. Animals and Housing

In trial 1, both young and old Long Evans female rats were employed. Young rats ranging in body weight from 74 to 115 g and old rats, retired as breeders, ranging in body weight from 244 to 336 g were grouped into 4 weight group replications per age group. All rats were placed on the low Ca (0.16%) depletion diet and fed a maximum of 17 g/day. After 7 weeks on the depletion diet, 12 rats from each age group were sacrificed. The remaining rats were placed on the 10 repletion diets by random assignment of each weight group and fed for 6 weeks at which time they were sacrificed.

In trial 2, both young and old Long Evans female rats were employed. Young rats ranging in body weight from 49 to 70 g and old rats, retired as breeders, ranging in body weight from 216 to 313 g were placed on a control diet for 14 days. At the end of this control period, 6 rats from each age group were sacrificed. The remaining rats were grouped into 4 weight group replications per age group and then each weight group was assigned randomly to the 8 diet treatments. A maximum of 16 g/day was fed for 7 weeks at which time all

rats were sacrificed.

In both trials, rats were housed individually in stainless steel, wire bottomed cages in a temperature (24-26°C) and humidity (55-60%) controlled room. Artificial illumination was regulated at 12 hours per day. All rats were fed and handled at the same time each day. During the first 3 days of each trial, a 1:1 mixture of Purina Rat Chow and purified diet was fed in order to accustom the rats to the purified diet. After this time, they were fed only the purified diet. Deionized distilled water was supplied ad libitum, and rat weights were recorded every other week. At the beginning of each trial all rats were weighed for 3 consecutive days and the average of these weighings served as the starting weight. On the starting day of each trial, the diet for each rat was placed in a plastic container and kept in the refrigerator at a temperature just above freezing for the duration of the experimental period. The diets were provided fresh to the rats each day.

C. Method of Sacrifice and Tissue Recovery

In all trials, rats were sacrificed after the last feeding day and were weighed before sacrifice. Each was anesthetized with ether until loss of righting reflex, lack of response to painful stimuli and depression of respiration were observed. Animals were then bound in dorsal recumbency to a surgical table and an ether nose cone was used to maintain the surgical stage of anesthetization. A T-incision was made to expose the abdominal cavity by adjoining a

ventral midline incision from pubis to xyphoid with both a left and right paracostal incision. The right and left cranial epigastric arteries were clamped with hemostats to prevent blood loss and then the falciform ligament of the liver was severed to reflect the liver and expose the diaphragm. A 1 cm incision was made in the left side of the diaphragm to expose the heart and blood was collected by cardiac puncture into a vacuum tube for determination of serum HP, Ca, Mg, cholesterol and protein.

After death by exsanguination, the animal was dissected to recover tissue samples for laboratory analyses. The left kidney and left half of the heart were removed and stored in the freezer until mineral analysis. The left femur was removed and frozen until density and radiographic measurements and mineral analyses. The right kidney, the remainder of the heart and the right femur were removed and fixed in 10% buffered formalin for histopathological section.

D. Laboratory Analysis

1. Diets

a. Dry matter determination

The amount of moisture or moisture-free matter is determined by loss of moisture in oven drying of chemically stable materials (138). Approximately 10 g samples of the basal, depletion, control and each test diet were analyzed for dry matter content by the following procedure:

1. Weigh (to the nearest 0.1 mg) the material to be tested into a tared crucible or drying dish.

2. Place the sample in an oven controlled at 105°C and dry overnight (to constant weight).

3. Cool in a desiccator to room temperature and weigh.

4. Calculate the percent dry matter as follows:

$$\% \text{ D.M.} = \frac{\text{dry sample weight}}{\text{wet sample weight}} \times 100$$

b. Crude protein determination

Samples of each diet were analyzed for N by the macro-Kjeldahl method, in which organic and inorganic N are reduced to ammonium sulfate in the presence of sulfuric acid and a catalyst (139). The ammonium sulfate is subsequently decomposed by 45% sodium hydroxide and the ammonia thus liberated is distilled into a 4% boric acid solution. The quantity of ammonia distilled into the boric acid is determined by titration with standardized sulfuric acid.

A 2 g dry sample of each diet was analyzed by the following procedure:

1. Add to each sample about 10 g of a potassium sulfate-cupric sulfate catalyst (7% $CuSO_4$ in K_2SO_4).

2. Add 25 ml of N-free concentrated sulfuric acid.

3. Digest for 30 minutes after mixture has cleared, then cool.

4. Dilute samples with 250 ml distilled water.

5. Add 80 ml of 45% N-free sodium hydroxide plus a few pellets of mossy zinc (3-5 g) and distill into a 4% boric acid solution.

6. Titrate the distillate to the red-orange end point of methyl red indicator (0.1% methyl red in alcohol) with 0.1 N H_2SO_4.

7. Calculate the percentage of crude portein as follows:

$$\%N = \frac{\text{ml } H_2SO_4 \times \text{Normality of } H_2SO_4 \times 14}{1000 \times \text{sample weight (g)}} \times 100$$

$$\%C.P. = \%N \times 6.25$$

c. Diet digestion and mineral analyses

The mineral content of biological materials can be determined by atomic absorption spectrophotometry (AAS). Biological materials are prepared for AAS by wet oxidation of all organic components with mineral acid. The inorganic residue is then analyzed. The following procedure was used:

1. Dry 3.0 g samples of diets for 24 hours at 105°C and determine the dry weight of each sample.

2. Prepare diet samples for mineral analysis by wet ashing with 5 ml of concentrated nitric acid and 2 ml of concentrated perchloric acid per g of dry sample (241).

3. Digest in acid washed beakers on a hot plate maintained at 300°C until clearing of samples occurs.

4. Dilute samples to 25 ml in volumetric flasks with deionized distilled water. For Ca and Mg analyses, further dilute the samples 500X with 1% lanthanum-oxide solution.

5. Analyze the digested samples for Fe, Mn, Zn, Cu, Ca and Mg by AAS using a Perkin-Elmer model 305-A

spectrophotometer (241).

6. The working ranges of standards (4% nitric acid) for the trace elements are: Fe, 1.0 to 5.0 ppm; Mn, 1.0 to 5.0 ppm; Zn, 1.0 to 5.0 ppm; Cu, 0.2 to 1.0 ppm. The working ranges of standards (1% lanthanum) for Ca and Mg are: Ca, 0.5 to 2.5 ppm; Mg, 0.1 to 0.5 ppm. Standard solutions against which samples are read are made from stock reagents.

The concentrations of P in the diet samples were determined by the ammonium molybdate method of Fiske and Subbarow (98), as follows:

1. Wet ash 3 g of the sample in acid and dilute to 25 ml in a volumetric flask with deionized distilled water as in the AAS mineral analysis described above.

2. Place a 0.1 ml aliquot of the diluted digested sample into a 10 ml volumetric flask.

3. Add 1.0 ml diammonium - molybdate solution and 0.4 ml aminonaphtholsulformic acid (98).

4. Bring the volume up to 10 ml and let stand for 5 minutes.

5. Measure absorbancy at 660 mu in a Bausch and Lomb Spectronic 20 against standard solution diluted from a stock solution to give a range from 2 to 10 ppm P.

d. Dietary pH determination

Dietary pH was determined by the method of Ali and Evans (5). Ten g samples of each diet were suspended in

90 ml deionized water and stirred, left 15 minutes to attain equilibrium and then restirred. The pH values were determined immediately on the equilibrated suspensions with a Beckman pH meter equipped with a combination electrode.

2. Serum

a. Serum minerals

The serum was analyzed for Ca and Mg. In a 10 ml volumetric flask, 0.2 ml of serum was brought to volume with a 1% lanthanum-oxide solution. This solution was then aspirated in a Perkin-Elmer model 305-A atomic absorption spectrophotometer (241).

Standard solutions against which samples were read were made from stock reagents. The working ranges for the standards (1% lanthanum) were 0.5 to 2.5 ppm for Ca and 0.1 to 0.5 ppm for Mg.

b. Serum cholesterol

Serum cholesterol was quantitated by the color reaction technique of Seary and Berquist (276). Into a 5 ml test tube was added 0.1 ml serum and 0.9 ml absolute ethanol-acetone (1:1) mixture. The tube was stoppered, mixed and centrifuged at 2000 rpm for 10 minutes. Then 0.5 ml clear supernatant of serum, 5.0 ml glacial acetic acid and 3.5 ml sulfuric acid color reagent (1 part 10% $FeCl_3$ in concentrated phosphoric acid made to 100 parts with concentrated sulfuric acid) were added to photometer tubes and shaken to mix the contents completely. After cooling for 15 minutes, the tubes were read in a Bausch and Lomb Spectronic 20 at 560 mu.

Standards ranging from 20 to 140 mg cholesterol per 100 ml were prepared and the resulting linear regression equation was used to calculate the cholesterol of samples in mg/100 ml of serum.

c. Serum hydroxyproline

Serum free HP was spectrophotometrically determined by the method of Bergman and Loxley (36). The following reagents were prepared:

A. Acetate-citrate buffer pH 6.

57 g sodium acetate ($3H_2O$), 37.5 g trisodium citrate ($2H_2O$), 5.5 g citric acid and 385 ml isopropanol are made up to 1 liter and is stable for long periods.

B. Oxidant solution.

A 7% aqueous solution (w/v) of chloramine T is prepared and can be stored for weeks.

C. Just before use, solutions A and B are mixed in the ratio of 4:1.

D. Ehrlich's reagent.

A solution of p-dimethylaminobenzaldhyde in 60% perchloric acid: 2 g of aldehyde is dissolved in 3 ml of acid and kept in a dark bottle. The solution is stable for several weeks.

E. Analyzed reagent isopropanol.

Just before use, D and E are mixed in a ratio of 2:13 to a final volume of 15 ml.

F. Hydroxproline standard solution.

A standard solution of l-hydroxyproline is prepared by dissolving 1 mg of the amino acid in 10 ml 0.001 N HCl (to prevent bacterial growth). A 10 ppm stock solution is then made from this 100 ppm solution.

Serum protein was precipitated by mixing 2 ml serum and 2 ml of 10% TCA and centrifuging at 2000 rpm. One ml of supernatant was taken for analysis and neutralized with 10, 1.0 and 0.1 N KOH to a lemon-yellow phenol red color in a graduated test tube. The volume was brought up to 4.5 ml with isopropanol. After gentle mixing, 0.5 ml of oxidant solution was added. After 5 minutes, 5 ml of Ehrlich's reagent was added (up to volume of 10 ml). The mixture was then gently mixed, kept 17-18 hrs at room temperature and read at 558 mu in a Bausch and Lomb Spectronic 20 against standard concentrations of 0.1, 0.2, 0.3 ppm. To prepare the standards, proper aliquots of the 10 ppm stock solution were brought up to a volume of 1 ml with deionized distilled water and then subjected to the same procedure as the 1 ml serum/TCA supernatant. The free HP of samples was calculated in ug/100 ml of serum using the standard linear regression equation.

d. Serum total protein

Serum total protein can be colorimetrically determined using a modification of Folin-Ciocalteau reagent (295). The extremely high sensitivity of this reagent makes it valuable for detecting the very low protein concentrations in highly diluted samples. The procedure of Bouering et al.

(53) was used. The following reagents were prepared:

1. Reagent A - 1 ml of 3.3% sodium-potassium tartrate ($NaKC_4H_4O_6 \cdot 4H_2O$) plus 1 ml of 1.25% copper sulfate ($CuSO_4 \cdot 5H_2O$) is brought to a volume of 100 ml with 2.5% sodium carbonate (Na_2CO_3).
2. Reagent B - commercial 2 N solution of Folin-Ciocalteau Reagent is diluted with deionized distilled water to be 0.5 N acid (1:4).
3. Stock standard - 6.0 g crystaline bovine albumin is brought to a volume of 100 ml with water. Then 0.1 ml of this solution is diluted to 6.0 ml with deionized distilled water to give a stock standard containing 1000 ug protein/ml.

Working standards of 3.0, 4.5, 6.0, 7.5 and 9.0 g protein/100 ml were prepared by taking 0.05, 0.075, 0.1, 0.125 and 0.15 ml, respectively, of the stock standard and proceeding as described for the samples.

The samples were prepared by securing clear unhemolyzed serum and diluting it 60X (0.1 ml in 6.0 ml) with deionized distilled water. To 0.1 ml of the diluted samples, 0.4 ml water was added (or the necessary amount in case of standards) up to 0.5 ml then 0.5 ml 1 N NaOH was added and mixed. Without longer delay (not more than a few minutes) 4.0 ml of reagent A was added rapidly in one portion and mixed. After standing for 10 minutes at room temperature, 1 ml of reagent B was added and mixed. After standing for 30 minutes at room temperature, the samples and standards were read at

600 mu in a Bausch and Lomb Spectronic 20 using a red filter and proper lamp. Serum total protein of the samples was calculated in g/100 ml of serum using the standard linear regression equation.

3. Soft Tissues

The whole left kidneys and half of each heart were digested for mineral analyses by the procedures described for diets in section D.1.c. All of the tissues were dried at 105°C for 24 hours, then wet ashed in acid washed beakers at 300°C until the samples cleared. Kidneys were diluted to 50 ml in a volumetric flask, whereas heart was diluted to 10 ml. A further 25X dilution was made for heart and kidney Mg and young kidney Ca determinations. All dilutions were made with 1% lanthanum. All diluted digestion samples were then read for Ca and Mg in a Perkin-Elmer model 305-A spectrophotometer.

Standard solutions against which the samples were read were made from stock reagents. Standards for the initial dilution samples contained 4.0% nitric acid and 1% lanthanum by volume. Standards for the further 25X diluted samples contained 1% lanthanum by volume. The working ranges of the standards were 0.5 to 2.5 ppm for Ca and 0.1 to 0.5 ppm for Mg.

4. Femur

a. Volume and gravimetric density

The frozen left femur was thawed and the adherent soft tissue manually removed using pointed scissors

and cheesecloth. To prevent excessive dehydration, the cleaned femur was immediately weighed on a Mettlar balance to the nearest 0.1 mg. Without further delay the femur was suspended on a perforated metal tray by fine copper wire on another Mettlar balance and weighed in water (to the nearest 0.1 mg). This latter balance was pre-zeroed with the metal tray, suspended in water.

The weight in air (WA) and weight in water (WW) were used to compute femur volume (V) in cc and gravimetric density or specific gravity (SG) in g/cc as follows (19):

$$V = WA - WW$$

$$SG = WA/V$$

b. Radiographic measurements

Following weight, volume and density measurements, the left femurs were radiographed on a Picker GX-1050 x-ray machine with a Dynamax 69B x-ray tube, a focal spot size of 0.6 mm, a filtration of 3.5 mm aluminum equivalent and a collimated field size of approximately 8 x 10 mm.

The exposure factors were 200 mA, 0.7 seconds, 48 kV and a focal film distance of 40 inches.

Kodak RPM X-omat Rapid Processing Mammography film was used. The film was processed for 90 seconds in a Picker Diplomat Automatic Processor using Kodak RP Developer and Fixer.

The resultant femur radiographs were individually magnified on a Wilder Micro Projector (Opto-metric Tools, Inc., New York, N.Y.) using a 20X objective lens and

projected onto an 8 1/2 x 11 inch sheet of white bond paper. The diaphyseal subperiosteal and endosteal cortical margins were traced using a sharpened number 2 2/4 lead pencil. The following middiaphyseal radiographic measurements (55) were taken with calipers (to the nearest 0.001 mm) from the tracings at the point of narrowest diameter: total tubular bone width (T), medullary cavity width (M) and both cortical thicknesses (C_1, C_2). From these measurements total cortical thickness (C), cortical area (CA), percent cortical area (PCA) and cortical index (CI) within the anatomical bone envelope were calculated as follows (55):

$$C = C_1 + C_2$$

$$CA = .785\ (T^2 - M^2)$$

$$PCA = 100\ \left(\frac{T^2 - M^2}{T^2}\right)$$

$$CI = {}^{C}/_{T}$$

c. Mineral analyses

Following radiographic measurements, the left femur from each carcass was dried in a forced draft oven at 105°C for 48 hours. They were then ether extracted for 24 hours and again dried for 8 hours. The dried femurs were ashed in previously weighed beakers in a muffle oven at 550°C for 24 hours.

In Trial 1 the ash was dissolved in 5 ml HCl heated on a hot plate at 100°C. The femur ash solutions were diluted to 50 ml with deionized distilled water and then further diluted with 1% lanthanum 500X for Ca and 50X for Mg determinations. Mineral determinations were made in an atomic

absorption spectrophotometer and read against standards as described in section D.1.c for diet analyses.

In Trial 2, the ash was dissolved in 20 ml of a gallium buffer solution (100 ppm gallium in 3 N HCl). Mineral determinations were made in a Jarrell-Ash Model 750 Atomocomp (149) and read against standards prepared in the same gallium buffer solution from stock standard solutions. The mineral determinations made and the working ranges of standards used were as follows: Ca, 500-5000 ppm; P, 250-2500 ppm; Mg, 100-1000 ppm; Na 50-500 ppm; K, 250-2500 ppm; Cu, 5-50 ppm; Fe, 5-50 ppm; Mn, 10-100 ppm; and Zn, 5-50 ppm.

5. Histopathological Study

a. Soft tissues

In both trials the whole right kidney and half of the heart were removed from each rat carcass and fixed in a 10% buffered formalin solution (Table 12) for a minimum of 24 hours. After fixation, the soft tissues were embedded in paraffin blocks consisting of Tissue Prep No. T-610 (The Fisher Scientific Co., Chemical Manufacturing Div., Fair Lawn, N. J.) with a melting point of 61.0°C (±0.5°C). The paraffin blocks were cut into 6 micron thick sections on a Spencer 820 microtome (Arthur H. Thomas, Philadelphia, Pa.) and the sections mounted on microscope slides as described in the Manual of Histologic Staining Methods of the AFIP (198). The slides were then dried in an oven at 56°C for 20 minutes and stained with Von Kossa's method for Ca (206) as described in Table 13. Following staining the slides were

covered by cover slips and sealed with Permount histological mounting medium (Fisher Scientific Co.).

b. Femur

In both trials the right femur was removed from each rat carcass and fixed in a 10% buffered formalin solution for a minimum of 24 hours. After fixing, the femurs were manually cleaned of the adhering soft tissue and decalcified for 48 hours as described in Table 12. The decalcified femurs were then trimmed into a longitudinal section of the proximal end, and a cross section of middiaphysis. The trimmed sections were dehydrated and cleared on an Auto Technicon Tissue Processor Model 2A (The Technicon Co., Chauncy, N.Y.) as described in Table 12. The prepared specimens were then embedded in paraffin, cut into 6 micron thick sections, mounted on microscope slides and dried as previously described. The mounted specimens were stained with routine Delafield's hematoxylin and eosin stain (198) as described in Tables 14 and 15. Following staining the slides were covered and sealed as previously described.

E. Statistical Analysis of Data

The data in these experiments were statistically evaluated by analysis of variance and regression analysis (286).

Table 1. Trial 1: The experimental design.

Acidity[a]	Protein	Age					
		Young % Ca			Mature % Ca		
		0.22	0.48	0.78	0.22	0.48	0.78
	%						
	9	(1)[b,c]	*	(4)	(6)	*	(9)
A	18	*	(3)	*	*	(8)	*
	36	(2)	*	(5)	(7)	*	(10)
	9	(1)	*	(4)	(6)	*	(9)
N	18	*	(3)	*	*	(8)	*
	36	(2)	*	(5)	(7)	*	(10)

[a] Acid added (A), natural (N).

[b] The diet number.

[c] Four replications per diet.

* Incomplete factorial.

Table 2. Composition of basal diet[a]

Constituents	%
Sucrose	30.0
Starch	30.0
Casein[b]	20.0[c]
D-L Methionine	0.2[c]
Corn Oil[d]	5.0
Vitamin Mixture	2.0
Micromineral Mixture	0.036
Macromineral Mixture	12.764

[a] Adapted from: Evans, J. L. and R. All, 1967. J. Nutr. 92:4, 417-424.

[b] Casein is 90.9% protein.

[c] Basal diet was 18% protein by analysis.

[d] Santoquin (Monsanto Cehmical Co.) added to corn oil to make 0.01% in diet.

Table 3. Composition of vitamin mixture contained in basal diet.

The vitamins mixed with dextrose supplied the following per 100 g of diet:[a,b]

Constituents	Amount
	mg
Vitamin A	9.0[c]
Vitamin D	0.5[d]
α-tocopherol	10.0
Ascorbic Acid	90.0
Inositol	10.0
Choline Chloride	150.0
Riboflavin	2.0
Menadione	4.5
p-Aminobenzoic Acid	10.0
Niacin	9.0
Pyridoxine·HCl	2.0
Thiamine	2.0
Ca Pantothenate	6.0
Biotin	0.04
Folic Acid	0.18
Vitamin B_{12}	0.003

[a] NRC requirements were met (National Research Council Committee on Animal Nutrition. 1972. Nutrient requirements of laboratory animals, pub. 2028-X. National Academy of Sciences - National Research Council, Washington, D.C., pp. 56-93).

[b] Computed on a dry basis.

[c] 1800 I.U.

[d] 1800 I.U.

Table 4. Composition of micromineral mixture contained in basal diet.

The micromineral mixture supplied the following minerals per 100 g of diet (supplied in the form and amount of indicated compound):[a,b]

Mineral (Form)	Amount by Calculation		Amount by Analysis	
	Mineral (Compound)		Trial 1	Trial 2
	mg	(mg)	ppm	ppm
Iron (Fe_2O_3)	8.58	(12.27)	88.9	84.3
Manganese ($MnCO_3$)	5.5	(11.5)	55.3	55.8
Zinc ($ZnCO_3$)	5.0	(9.59)	53.3	50.2
Copper ($CuCO_3$)	1.17	(2.27)	11.4	11.1
Cobalt ($CoCO_3$)	0.04	(0.08)	*	*
Molybdenum ($Na_2MoO_4 \cdot 2H_2O$)	0.06	(0.15)	*	*
Iodine (KI)	0.02	(0.026)	*	*
Selenium (H_2SeO_3)	0.01	(0.016)	*	*
Fluoride (NaF)	0.001	(0.002)	*	*

[a] NRC requirements were met (National Research Council Committee on Animal Nutrition. 1972. Nutrient requirements of laboratory animals, pub. 2028-X. National Academy of Sciences - National Research Council, Washington, D.C., pp. 56-93).

[b] Computed on a dry basis.

* Not analyzed due to low concentrations.

Table 5. Composition of macromineral mixture contained in basal diet.

The macromineral mixture supplied the following per 100 g of diet:[a]

Constituents	Amount
	mg
Calcium Acetate	415
Potassium Phosphate, Monobasic	870
Sodium Phosphate, Dibasic	927
Potassium Chloride	95
Magnesium Chloride	313
Magnesium Acetate	123
Chromic Oxide	250
Nonnutritive Bulk	*

[a] Computed on a dry basis.

[b] The macromineral mixture supplied the following per 100 g of diet on a dry basis:[c]

	Calculated	Amount by Analysis Trial 1	Trial 2
	mg	%	%
Sodium	300	0.30	0.30
Potassium	300	0.30	0.30
Phosphorus	400	0.44	0.43
Chloride	154	0.14	0.15
Magnesium	51	0.041	0.053
Acetate	340	-----	-----

[c] NRC requirements were met.

* See Table 6.

Table 6. Trial 1: Calcium and non-nutritive bulk (NNB) added to 100 g of diet.

Diets	NNB[a]	Ca Lactate[b]	$CaCO_3$	Calcium Supplied[c]	
				Calculated	By Analysis
	mg	mg	mg	%	%
1,4,6,9	9457	--	314	0.22	0.22
3,8	8807	--	964	0.48	0.48
2,5,7,10	8058	--	1713	0.78	0.78
Depletion	9209	562	--	0.16	0.17

[a] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[b] Calories from lactate were balanced with sucrose by replacing non-nutritive bulk.

[c] Includes 415 mg of Ca acetate per 100 g of basal diet.

Table 7. Trial 1: Sucrose, starch and protein supplied to the diets[a]

Diets	Sucrose	Starch	Casein	D-L Methionine	Protein by Analysis
	%	%	%	%	%
1,2,6,7	35.05	35.05	10	0.1	9
4,5,9,10	19.90	19.90	40	0.4	36

[a] Diets 3,8 and depletion contained the basal levels.

Table 8. Trial 1: Percent dry matter and pH of repletion diet.

Diet	ml Added per 100 g of Diet		Final Analysis	
	2 N HCl[a]	H_2O[b]	% Dry Matter	pH
1	4.35	3.80	89.2	5.2
2	7.65	0.50	89.0	5.2
3	6.45	1.50	88.6	5.2
4	3.60	3.50	88.3	5.2
5	7.35	*	88.3	5.2
6	N[c]	7.84	89.5	6.0
7	N	7.62	89.0	6.0
8	N	7.51	88.6	5.8
9	N	6.83	88.6	5.4
10	N	6.83	88.8	5.6

[a] Milliliters of 2 N HCl added to 100 g of diet = 1/2 x ml of 0.1 N HCl needed to titrate 10 g air dry weight of that diet in 90 ml of deionized distilled water to pH 5.2.

[b] Milliliters of deionized distilled water added to 100 g of diet = (1/desired final % dry matter) ([100 g diet x initial % dry matter] + [.07292 x ml 2 N HCl added to 100 g diet]) - 100 g diet - ml 2 N HCl added to 100 g diet.

[c] No acid was added to natural (N) diets.

* Final % dry matter of all diets was equilibrated to diet 5 which had the lowest initial % dry matter.

Table 9. Trial 2: The experimental design.

Age	% Ca	pH Level 5.0	5.8	6.6	7.4
Mature	0.48	(1)[a,b]	(2)	(3)	(4)
	0.78	(5)	(6)	(7)	(8)
Young	0.48	(1)	(2)	(3)	(4)
	0.78	(5)	(6)	(7)	(8)

[a] The diet number.

[b] Four replications per diet.

Table 10. Trial 2: Calcium and non-nutritive bulk (NNB) added to 100 g of diet.

Diets	NNB[a]	$CaCO_3$	Calcium Supplied[b]	
			Calculated	By Analysis
	mg	mg	%	%
Control	8807	964	0.48	0.48
1,2,3,4	8807	964	0.48	0.48
5,6,7,8	8058	1713	0.78	0.78

[a] Alphacel, Nutritional Biochemical Corporation, Cleveland.

[b] Includes 415 mg of calcium acetate per 100 g of basal diet.

Table 11. Trial 2: Percent dry matter and pH of test diets.

Diet	ml Added per 100 g of Diet			Final Analysis	
	2 N HCl[a]	2 N NaOH[a]	H_2O[b]	% Dry Matter	pH
1	5.7	---	3.14	87.4	5.0
2[c]	---	---	8.43	88.2	5.8
3	---	4.7	4.05	88.2	6.6
4	---	9.0	*	87.8	7.4
5	6.5	---	2.38	87.8	5.0
6[c]	---	---	8.43	88.2	5.8
7	---	4.7	4.05	88.2	6.6
8	---	9.0	*	87.8	7.4

[a] Milliliters of 2 N HCl or 2 N NaOH added to 100 g of diet = 1/2 x ml of 0.1 N HCl or 0.1 N NaOH needed to titrate 10 g air dry weight of that diet in 90 ml deionized distilled water to desired pH level.

[b] Milliliters of deionized distilled water added to 100 g of diet = (1/desired final % dry matter) ([100 g diet x initial % dry matter] + [.07292 x ml 2 N HCl added to 100 g diet] + [.079994 x ml 2 N NaOH added to 100 g diet]) - 100 g diet - ml 2 N HCl added to 100 g diet - ml 2 N NaOH added to 100 g diet.

[c] Natural acidity of diets 2 and 6 was pH 5.8.

* Final % dry matter of all diets was equilibrated to diets 4 and 8 which had the lowest initial % of dry matter.

Table 12. Solutions used in the fixing, decalcification, dehydration and clearing of histological specimens (198).

1. Fixing

10% Buffered Formalin:

37-40% formalin	100 ml
distilled H_2O	900 ml
sodium phosphate monobasic	4.0 g
sodium phosphate dibasic (anhydrous)	6.5 g

2. Decalcification

Formic Acid - Sodium Citrate - Decal Solution:

Solution A

sodium citrate	50 g
distilled H_2O	250 ml

Solution B

formic acid, 90%	125 ml
distilled H_2O	125 ml

Procedure

a. Mix solutions A and B in equal portions.

b. Place calcified speciments in large quantities of formic acid-sodium citrate solution until decalcification is complete.

c. Place solution under vacuum to hasten decalcification.

d. When decalcification is complete, wash in running water from 4-8 hours.

3. Dehydration and Clearing

Immerse specimens in the following solutions successively:

95% alcohol	1 hr
absolute alcohol (4X)	2 hr each
absolute alcohol and xylene (50-50 mixture)	1 hr
xylene (2X)	2 hr each

Table 13. Von Kossa's method for calcium (206).

Fixation: 10% buffered formalin

Technique: cut paraffin sections at 6 microns

Solutions:

5% silver nitrate solution

silver nitrate	5.0 g
distilled H_2O	100 ml

5% sodium thiosulfate (Hypo) solution

sodium thiosulfate	5.0 g
distilled H_2O	100 ml

Nuclear fast red (Kernechtrot) solution

Dissolve 0.1 g nuclear fast red in 100 ml of 5% solution of aluminum sulfate with aid of heat. Cool, filter, add grain of thymol as a preservative.

Staining Procedure:

1. Deparaffinize and hydrate tc H_2O, 2X: 2,3 minutes
2. Silver nitrate solution for 60 minutes exposed to direct sunlight
3. Rinse in distilled H_2O, 2X: 2,3 minutes
4. Sodium thiosulfate solution for 2 minutes
5. Rinse well in distilled H_2O, 3X: 3,3,3 minutes
6. Counterstain in nuclear fast red solution for 5 minutes
7. Rinse in distilled H_2O, 2X: 2,3 minutes
8. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, 2 changes each
9. Mount with Permount

Results:

Calcium salts - black

Nuclei - red

Cytoplasm - light pink

Table 14. Routine Delafield's H and E staining solutions (198).

A. Delafield's Hematoxylin:

hematoxylin (crystals)	8 g
alcohol, 95%	50 ml
ammonium alum (ammonium aluminum sulphate)	75 g
distilled H_2O	800 ml

1. Let above solutions stand separately overnight.
2. Mix, expose to light and air 3-4 days. Shake occasionally.
3. Filter.
4. Add glycerin (200 ml) and 95% alcohol (200 ml).
5. Let stand in light until deep purple.
6. Filter, store in dark bottle, age 6-8 weeks.

B. Eosin B Counterstain:

stock 50% alcohol (from 95%)	100 ml
800 ml 95% alcohol	
200 ml distilled H_2O	
Eosin B	0.5 g

When ready to use, add glacial acetic acid, 1 drop per 200 ml.

C. Parlodion, 0.5%:

absolute alcohol	250 ml
ether	250 ml
parlodion strips	2.5 g

D. Acid Alcohol:

70% alcohol	900 ml
concentrated HCl	10 ml
mix well	

E. Ammonia H_2O:

distilled H_2O	130 ml
NH_3OH	2 drops

Table 15. Routine Delafield's H and E staining procedure (198).

Immerse specimens in the following solutions, successively:

1. Xylene, 2X: 5,5 minutes.
2. Absolute alcohol, 2X: 5,5 minutes.
3. Parlodion, 5 minutes. Drain 5 minutes.
4. 80% alcohol, 5 minutes. Flush with tap water, 5 minutes.
5. Delafield's hematoxylin, 15 minutes
6. Water, 1 dip.
7. 1% acid alcohol, 1 dip.
8. Water, 3 minutes.
9. Ammonia H_2O, 2 minutes. Flush with tap water, 10 minutes.
10. Eosin B, 1-2 minutes.
11. 95% alcohol, 2X: 3,3 dips.
12. Absolute alcohol, 2X: 5,5 minutes.
13. Xylene, 2X: 5,5 minutes. Mount with permout.

Results:

Nuclei - blue with some metachromasia.

Cytoplasm - various shades of pink identifying different tissue components.

IV. RESULTS

A. Final Body Weight and Weight Gain

1. Trial 1

Diet treatment had no significant effect on final body weight and average daily gain of either young or old rats in Trial 1.

The range of final body weights of young Trial 1 rats were as follows: initial, 74-115 g; depletion, 198-271 g; repletion, 248-315 g. The mean final body weights (± standard error of the mean) of young Trial 1 rats were: initial, 99 ± 1 g; depletion, 241 ± 3 g; repletion, 272 ± 3 g. The average daily weight gain of young Trial 1 rats was 2898 mg during the 7 week depletion period and 633 mg during the 6 week repletion period.

The range of final body weights of old Trial 1 rats were as follows: initial, 244-336 g; depletion, 276-382 g; repletion, 273-360 g. The mean final body weights (± S.E.) of old Trial 1 rats were: initial, 286 ± 4 g; depletion, 323 ± 4 g; repletion, 322 ± 4 g. The average daily weight gain of old Trial 1 rats was 959 mg during the 7 week depletion period and -24 mg during the 6 week repletion period.

2. Trial 2

Diet treatment had no significant effect on final body weight and average daily gain of either young or old rats in Trial 2.

The range of final body weights of young Trial 2 rats were as follows: initial, 49-70 g; standardization period, 91-117 g; test period, 200-259 g. The mean final body weights (± S.E.) of young Trial 2 rats were: initial 60 ± 1 g; standardization, 103 ± 1 g; test, 230 ± 3 g. The average daily weight gain of young Trial 2 rats was 3071 mg during the 2 week standardization period and 2592 mg during the 7 week test period.

The range of final body weights of old Trial 2 rats were as follows: initial 216-313 g, standardization, 234-338 g; test, 268-373 g. The mean final body weights (± S.E.) of old Trial 2 rats were: initial, 260 ± 4 g; standarization, 281 ± 4 g; test, 306 ± 4 g. The average daily weight gain of old Trial 2 rats was 1500 mg during the 2 week standardization period and 510 mg during the 7 week test period.

B. Femur Radiographic Measurements

1. Trial 1

The interactions of age and diet treatment on femur radiographic measurements in Trial 1 are summarized in Tables 16-19.

In Trial 1, the 6 week period of Ca repletion with graded levels of diet Ca (0.22, 0.48, 0.78%) resulted in both increased subperiosteal and endosteal bone deposition in young growing rats, compared to the 7 week Ca-depleted (0.16%) controls. This was evidenced by a larger subperiosteal diameter (T) and a smaller medullary cavity diameter (M) in the repleted young rats than in the depleted young controls

(Table 16). The net result was a greater cortical thickness (C), cortical area (CA), percent cortical area (PCA) and cortical index (CI) in the repleted young rats than in the depleted young controls (Table 16). Calcium depletion-repletion had no significant effect on radiographic measurements of old rats in Trial 1 (Table 16).

In Ca-depleted young rats, both T and M increased with increased diet Ca (Table 17). The net result was a thinner C and reduced PCA and CI (Table 17). Total bone mass as measured by CA, however, was slightly increased by increased diet Ca in the Ca-depleted young rats (Table 17). Increased diet Ca had no significant effect on radiographic measurements of Ca-depleted old rats (Table 17).

In Ca-depleted young rats, both T and M increased with increased diet protein (Table 18). The net result was a slightly thinner C and reduced PCA and CI (Table 18). Total bone mass as measured by CA, however, was increased by increased diet protein in the Ca-depleted young rats (Table 18) Increased diet protein had no significant effect on radiographic measurements of depleted old rats (Table 18).

In Ca-depleted young rats, acid addition to the repletion diets resulted in greater endosteal resorption, but reduced subperiosteal deposition as evidenced by a slightly larger M and slightly smaller T (Table 19). The net results from greater diet acidity were a thinner C and reduced CA, PCA and CI with diet acid addition (Table 19). In Ca-depleted old rats, acid addition to the repletion diets

also resulted in slightly decreased subperiosteal deposition, but decreased endosteal resorption, as evidenced by both a smaller T and M (Table 19). The net results were still a thinner C and reduced CA (Table 19). Diet acid addition, however, had no effect on PCA and CI in the Ca-depleted old rat (Table 19).

2. Trial 2

The interactions of age and diet treatment on femur radiographic measurements in Trial 2 are summarized in Tables 20-22.

In Trial 2, at the end of the 7 week experimental period, the femurs of the young rats showed increased turnover at both bone surfaces, compared to the randomly selected non Ca-depleted controls. This was evidenced by both a larger T and M (Table 20). The net results were a thicker C and increased CA, PCA and CI (Table 20). There was no significant difference between the femur radiographic measurements of the randomly selected old control rats and the old rats at the end of the 7 week experimental period (Table 20).

In contrast to the Ca-depleted rats of Trial 1, increased diet Ca had no significant effect on femur radiographic measurements of either age group of the non Ca-depleted rats of Trial 2 (Table 21).

In Trial 2, the effects of increased diet acidity on femur radiographic measurements of the non Ca-depleted young rats were identical to those in the Ca-depleted young rats of Trial 1. With increased diet acidity, T was smaller

while M was larger (Table 22). The net results were a thinner C and reduced CA, PCA and CI (Table 22), just as in Trial 1. The effects of increased diet acidity on femur radiographic measurements of the non Ca-depleted old rats were also identical to that in the Ca-depleted old rats. With increased diet acidity, both T and M were smaller, C thinner and CA reduced, while PCA and CI remained unchanged in non Ca-depleted old rats (Table 22).

C. Femur Gravimetry and Mineral Composition

1. Trial 1

a. Femur gravimetry

The interactions of age and diet treatment on femur gravimetric measurements in Trial 1 are summarized in Tables 23-26.

In Trial 1, the 6 week period of Ca repletion with graded levels of diet Ca (0.22, 0.48, 0.78%) resulted in an increase in all femur gravimetric measurements in young growing rats (Table 23), compared to the 7 week Ca-depleted (0.16%) controls. The Ca depletion-repletion had no significant effect on any gravimetric measurement of old rats in Trial 1 (Table 23).

In Ca-depleted young rats, femur fat-free dry weight and ash both increased with increased diet Ca, while femur air dry weight increased from 0.22% to 0.48% diet Ca before plateauing at 0.48% diet Ca (Table 24). As a result, both fat-free dry weight and ash expressed as percent of air dry weight also increased, while ash expressed as a percent of

fat-free dry weight increased from 0.22% to 0.48% diet Ca before plateauing at 0.48% diet Ca (Table 24). With increased diet Ca, femur density (mg ash/cc) and specific gravity also increased in the Ca-depleted young rats while femur volume remained basically unchanged (Table 24). In Ca-depleted old rats, femur fat-free dry weight, ash and ash as a percent of fat-free dry weight increased significantly with increased diet Ca (Table 24). There was also a trend toward an increase in femur air dry weight, while fat-free dry weight and ash as percent of air dry weight increased from 0.22% to 0.48% diet Ca before plateauing at 0.48% diet Ca (Table 24). With increased diet Ca, femur density and specific gravity also increased, while femur volume remained basically unchanged in the Ca-depleted old rats (Table 24).

In Ca-depleted young rats, femur density and specific gravity increased linearly with increased diet protein, while all other gravimetric measurements remained unchanged (Table 25). In Ca-depleted old rats, increased diet protein had a quadratic effect on femur density and specific gravity, while all other gravimetric measurements remained unchanged (Table 25).

In Ca-depleted young rats, acid addition to the repletion diets resulted in a decrease in all femur gravimetric measurements (Table 26). In Ca-depleted old rats, only ash as a percent of air dry weight, density and specific gravity showed a significant decrease with diet acid addition (Table 26).

b. Femur mineral composition

The interactions of age and diet treatment on femur mineral composition in Trial 1 are summarized in Tables 27-30.

In Trial 1, the 6 week period of Ca repletion resulted in an increase in both femur Ca and Mg, but no change in those minerals expressed as percent in ash, in young growing rats, compared to the 7 week Ca-depleted controls (Table 27). Calcium depletion-repletion had no significant effects on femur mineral composition of old rats in Trial 1 (Table 27).

With increased diet Ca, femur Ca and Mg increased in both age groups of Ca-depleted rats, while no change was observed in those minerals when expressed as percent in ash (Table 28).

Increased diet protein (Table 29) or diet acid addition (Table 30) had no significant effects on femur mineral composition in either age group of Ca-depleted rats in Trial 1.

2. Trial 2

a. Femur gravimetry

The interactions of age and diet treatment on femur gravimetric measurements in Trial 2 are summarized in Tables 31-33.

In Trial 2, the 7 week experimental period resulted in an increase in all femur gravimetric measurements in non Ca-depleted young growing rats compared to the randomly selected controls (Table 31). In the non Ca-depleted old rats there was no significant difference in femur gravimetric

measurements between the experimental groups and the randomly selected controls (Table 31).

Increased diet Ca had the same effect on femur gravimetric measurements in both age groups of non Ca-depleted rats of Trial 2. Air dry weight and volume remained unchanged, while all other femur gravimetric measurements increased with increased diet Ca (Table 32).

In non Ca-depleted young rats, increased diet acidity had no effect on femur ash expressed as a percent of fat-free dry weight, but all other gravimetric measurements were significantly decreased (Table 33). In non Ca-depleted old rats, increased diet acidity had no effect on femur ash expressed either as a percent of fat-free dry weight or as a percent of air dry weight, but all other gravimetric measurements were significantly decreased (Table 33).

b. Femur mineral composition

The interactions of age and diet treatment on femur mineral composition in Trial 2 are summarized in Tables 34-36.

In Trial 2, the 7 week experimental period resulted in a decrease in femur P, Na, K, Ca and Mn expressed as percent in ash in non Ca-depleted young rats compared to the randomly selected controls (Table 34). There was no such effect on the femur composition of non Ca-depleted old rats (Table 34).

Increased diet Ca resulted in a decrease in K as a percent in ash in both age groups of non Ca-depleted rats (Table 35). Increased diet Ca had no significant effect on other

femur minerals expressed as percent in ash in either age group in Trial 2.

Increased diet acidity had no significant effect on femur minerals expressed as percent in ash in either age group of non Ca-depleted rats (Table 36).

D. Serum Composition

1. Trial 1

The interactions of age and diet treatment on serum composition in Trial 1 are summarized in Tables 37-40.

In Trial 1, the 6 week period of Ca repletion resulted in decreased serum hydroxyproline (HP) and increased serum cholesterol in both age groups of rats compared to the depleted controls, while serum Ca, Mg and protein remained unchanged in both age groups (Table 37).

In both age groups of Ca-depleted rats, increased diet Ca (Table 38) or protein (Table 39) resulted in a linear decrease in serum HP, a quadratic change in serum cholesterol, but no change in serum Ca, Mg and protein.

In both age groups of Ca-depleted rats, increased diet acidity resulted in increased serum HP and Mg, and decreased serum Ca, cholesterol and protein (Table 40).

2. Trial 2

The interactions of age and diet treatment on serum composition in Trial 2 are summarized in Tables 41-43.

In Trial 2, at the end of the 7 week experimental period, the serum HP of the young rats was lower than the randomly selected non Ca-depleted controls (Table 41). No other

significant differences in serum composition between experimental and control rats were seen in either age group (Table 41).

In both young and old non Ca-depleted rats, increased diet Ca resulted in decreased serum HP and cholesterol, but no change in serum Ca and Mg (Table 42).

In both young and old non Ca-depleted rats, increased diet acidity resulted in increased serum HP, and no change in serum Ca and Mg, while the effect on serum cholesterol was quadratic (Table 43).

E. Soft Tissue Mineralization

1. Trial 1

The interactions of age and diet treatment on soft tissue mineralization in Trial 1 are summarized in Tables 44-47.

In Trial 1, the 7 week period of Ca depletion with a Ca-deficient (0.16%), low Mg (0.04%) diet resulted in nephrocalcinosis in young growing rats as evidenced by mineral analyses of control kidneys (Table 44) and confirmed by histopathologic examination (Figure 1A). The depletion diet did not produce nephrocalcinosis in old rats (Figure 1B), or cardiac calcinosis in either age group (Table 44).

With increased diet Ca, heart Ca increased and Mg decreased in both age groups (Table 45). In Ca-depleted old rats, kidney Ca increased and Mg decreased with increased diet Ca (Table 45), although both Ca and Mg levels of the already calcified young kidneys were lower with increased

diet Ca (Table 45). The lower kidney Ca levels of young rats were confirmed by histopathologic examination (Figure 2A,B).

Increased diet protein per se had no significant effect on soft tissue mineral levels (Table 46).

In Ca-depleted old rats, acid addition to the repletion diets resulted in higher Ca and lower Mg levels in both the heart and kidney (Table 46). These mineral levels fell within normal ranges. In Ca-depleted young rats, diet acid addition resulted in a similar reciprocal effect on heart Ca and Mg levels. However, both Ca and Mg levels of the already calcified kidneys of Ca-depleted young rats were higher on the acid-added diets than on the natural diets (Table 47). The higher kidney Ca levels of young rats were confirmed by histopathologic examination (Figure 1C,D).

2. Trial 2

The interactions of age and diet treatment on soft tissue mineralization in Trial 2 are summarized in Tables 48-50.

The randomly selected controls of neither age group of Trial 2 rats had calcified soft tissues, as indicated by mineral analyses of the control hearts and kidneys (Table 48).

In Trial 2, the effects of increased diet Ca or acidity on soft tissue mineral levels of the non Ca-depleted old rats were identical to those in Trial 1. With increased diet Ca, Ca increased and Mg decreased in both heart and kidney (Table 49). With increased diet acidity, both heart and kidney Ca

increased and Mg decreased within the normal ranges (Table 50).

In the non Ca-depleted young rats of Trial 2, heart Ca increased and heart Mg decreased with increased diet Ca (Table 49) or increased diet acidity (Table 50), just as in the Ca-depleted young rats of Trial 1.

The low Mg level (0.05%) of the Trial 2 diets induced nephrocalcinosis in the non Ca-depleted young rats just as in the Ca-depleted young rats of Trial 1. In Trial 2 the effect of diet Ca on the severity of nephrocalcinosis in the young rats was identical to that in Trial 1. Increased diet Ca resulted in lower levels of both Ca and Mg in the calcified kidneys (Table 49). The lower kidney Ca levels were confirmed by histopathologic examination (Figure 2C,D). The effect of diet acidity on the severity of low diet Mg-induced nephrocalcinosis in the non Ca-depleted young rats of Trial 2 was different from the effect of diet acidity on kidneys of young rats already calcified by the low Mg, Ca-depletion diet of Trial 1. In Trial 2 both systemic acidosis and alkalosis resulted in lower levels of both Ca and Mg in the calcified kidneys (Table 50). The lower kidney Ca levels were confirmed by histopathologic examination (Figure 3A, B, C, D).

The low diet Mg-induced nephrocalcinosis of young rats in both trials was confined to the medullary region (Figure 4A). There was no glomerular involvement (Figure 4B) as calcification was confined to kidney tubules. The

calcification in these trials began in the basement membrane (Figure 4C), later involved the tubular epithelial cells and eventually led to the complete disintegration of the involved tubules (Figure 4D).

Table 16. Interaction of age and calcium depletion-repletion on femur radiographic measurements: Trial 1.

Treatment	Depleted		Repleted	
Age[a]	Young	Old	Young	Old
Measurement[b]				
T, mm	3.114 ± 0.020[c]	3.454 ± 0.041[d]	3.194 ± 0.019[e]	3.370 ± 0.028[f]
M, mm	2.130 ± 0.035	2.134 ± 0.049	2.048 ± 0.023	2.082 ± 0.029
C, mm	0.984 ± 0.024[c]	1.320 ± 0.023[d]	1.146 ± 0.007[c]	1.266 ± 0.014[d]
CA, mm^2	4.05 ± 0.08[c]	5.79 ± 0.11[d]	4.71 ± 0.03[c]	5.49 ± 0.07[d]
PCA, %	53.2 ± 1.1[c]	61.9 ± 1.1[d]	58.9 ± 0.5[e]	61.9 ± 0.05[f]
CI	0.316 ± 0.008[c]	0.383 ± 0.009[d]	0.359 ± 0.004[e]	0.383 ± 0.004[f]

[a] Values for each depleted and repleted age are means of 12 and 40 observations, respectively, ± standard error of the mean.

[b] Total subperiosteal diameter (T), medullary cavity diameter (M), cortical thickness (C), cortical area (CA), percent cortical area (PCA), cortical index (CI).

[c,d] Means having different superscripts in the same row under the same treatment are significantly different (P<.01).

[e,f] Means having different superscripts in the same row under the same treatment are significantly different (P<.05).

Table 17. Interaction of age and diet calcium on femur radiographic measurements: Trial 1.

Age	Young				Old			
Calcium, %[a]	0.22	0.48	0.78	± S.E.[b]	0.22	0.48	0.78	± S.E.
Measurement[c]								
T, mm	3.102	3.219	3.277[d]	0.019	3.357	3.403	3.365	0.028
M, mm	1.929	2.063	2.160[e]	0.023	2.070	2.114	2.078	0.029
C, mm	1.172	1.149	1.117[e]	0.007	1.264	1.289	1.256	0.014
CA, mm^2	4.63	4.76	4.76	0.03	5.44	5.58	5.49	0.07
PCA, %	61.3	58.8	56.6[d]	0.5	62.0	61.4	61.9	0.5
CI	.378	.358	.341[d]	.004	.384	.379	.384	.004

[a] Values for 0.22, 0.48 and 0.78% calcium are means of 16, 8 and 16 observations, respectively, averaged across 3 diet protein levels and 2 diet acidity conditions with no interactions.

[b] Standard error of the mean.

[c] Total subperiosteal diameter (T), medullary cavity diameter (M), cortical thickness (C), cortical area (CA), percent cortical area (PCA), cortical index (CI).

[d] Effect due to calcium is linear ($P<.01$).

[e] Effect due to calcium is linear ($P<.05$).

Table 18. Interaction of age and diet protein on femur radiographic measurements: Trial 1.

Age	Young				Old			
Protein, %[a]	9	18	36	± S.E.[b]	9	18	36	± S.E.
Measurement[c]								
T, mm	3.114	3.219	3.264[d]	0.019	3.355	3.403	3.367	0.028
M, mm	1.958	2.063	2.131[d]	0.023	2.073	2.114	2.074	0.029
C, mm	1.156	1.149	1.133	0.007	1.271	1.289	1.250	0.014
CA, mm^2	4.60	4.76	4.79[d]	0.03	5.42	5.58	5.52	0.07
PCA, %	60.5	58.8	57.4[e]	0.5	61.9	61.4	62.1	0.5
CI	.372	.358	.348[e]	.004	.383	.379	.385	.004

[a] Values for 9, 18 and 36% protein are means of 16, 8 and 16 observations, respectively, averaged across 3 diet Ca levels and 2 diet acidity conditions with no interactions.

[b] Standard error of the mean.

[c] Total subperiosteal diameter (T), medullary cavity diameter (M), cortical thickness (C), cortical area (CA), percent cortical area (PCA), cortical index (CI).

[d] Effect due to protein is linear ($P<.10$).

[e] Effect due to protein is linear ($P<.05$).

Table 19. Interaction of age and diet acidity on femur radiographic measurements: Trial 1.

Age	Young			Old		
Treatment[a]	Acid-Added	Natural	± S.E.[b]	Acid-Added	Natural	± S.E.
Measurement[c]						
T, mm	3.187	3.200	0.019	3.348	3.391	0.029
M, mm	2.062	2.035	0.023	2.067	2.097	0.029
C, mm	1.126[d]	1.165[e]	0.007	1.238[d]	1.294[e]	0.014
CA, mm^2	4.64[f]	4.78[g]	0.03	5.44[f]	5.54[g]	0.07
PCA, %	58.2[f]	59.6[g]	0.5	61.9	61.8	0.5
CI	.354[f]	.365[g]	.004	.384	.382	.004

[a] Values for each treatment are means of 20 observations, averaged across 3 diet Ca and 3 diet protein levels with no interaction.

[b] Standard error of the mean.

[c] Total subperiosteal diameter (T), medullary cavity diameter (M), cortical thickness (C), cortical area (CA), percent cortical area (PCA), cortical index (CI).

[d,e] Means having different superscripts in the same row under the same age are significantly different ($P<.05$).

[f,g] Means having different superscripts in the same row under the same age are significantly different ($P<.10$).

Table 20. Interaction of age and control versus test diet on femur radiographic measurements: Trial 2.

Treatment	Control		Test	
Age[a]	Young	Old	Young	Old
Measurement[b]				
T, mm	2.595 ± 0.057[c]	3.500 ± 0.078[d]	3.104 ± 0.018[c]	3.440 ± 0.031[d]
M, mm	1.756 ± 0.053[c]	2.190 ± 0.053[d]	2.029 ± 0.018	2.108 ± 0.028
C, mm	0.839 ± 0.032[c]	1.310 ± 0.037[d]	1.076 ± 0.011[c]	1.335 ± 0.013[d]
CA, mm^2	2.87 ± 0.14[c]	5.86 ± 0.27[d]	4.34 ± 0.05[c]	5.81 ± 0.09[d]
PCA, %	54.2 ± 1.5[c]	60.8 ± 0.8[d]	57.3 ± 0.4[c]	62.6 ± 0.5[d]
CI	0.323 ± 0.011[c]	0.375 ± 0.007[d]	0.347 ± 0.003[c]	0.389 ± 0.004[d]

a Values for each control and test age are means of 6 and 32 observations, respectively, ± standard error of the mean.

b Total subperiosteal diameter (T), medullary cavity diameter (M), cortical thickness (C), cortical area (CA), percent cortical area (PCA), cortical index (CI).

c,d Means having different superscripts in the same row under the same treatment are significantly different (P<.01).

Table 21. Interaction of age and diet calcium on femur radiographic measurements: Trial 2.

Age	Young			Old		
Calcium, %[a]	0.48	0.78	± S.E.[b]	0.48	0.78	± S.E.
Measurement[c]						
T, mm	3.101	3.108	0.018	3.441	3.439	0.031
M, mm	2.029	2.028	0.018	2.109	2.106	0.028
C, mm	1.072	1.080	0.011	1.335	1.333	0.013
CA, mm^2	4.32	4.35	0.05	5.82	5.81	0.09
PCA, %	57.1	57.4	0.4	62.6	62.5	0.5
CI	.346	.348	.003	.389	.388	.004

[a] Values for each treatment are means of 16 observations, averaged across 4 diet acidity conditions with no interaction.

[b] Standard error of the mean.

[c] Total subperiosteal diameter (T), medullary cavity diameter (M), cortical thickness (C), cortical area (CA), percent cortical area (PCA), cortical index (CI).

Table 22. Interaction of age and diet acidity on femur radiographic measurements: Trial 2.

Age	Young					Old				
Diet Acidity, pH[a]	5.0	5.8	6.6	7.4	± S.E.[b]	5.0	5.8	6.6	7.4	± S.E.
Measurement[c]										
T, mm	3.063	3.112	3.119	3.124[d]	0.018	3.369	3.404	3.455	3.532[d]	0.031
M, mm	2.044	2.041	2.027	2.002[d]	0.018	2.079	2.101	2.100	2.151[d]	0.028
C, mm	1.019	1.071	1.092	1.122[d]	0.011	1.290	1.311	1.355	1.381[d]	0.013
CA, mm^2	4.09	4.33	4.41	4.52[d]	0.05	5.52	5.66	5.91	6.16[d]	0.09
PCA, %	55.4	57.0	57.8	58.9[d]	0.4	62.0	62.4	63.0	62.9	0.5
CI	.333	.344	.350	.359[d]	.003	.385	.387	.392	.391	.004

[a] Values for each treatment are means of 8 observations, averaged across 2 diet Ca levels with no interaction.

[b] Standard error of the mean.

[c] Total subperiosteal diameter (T), medullary cavity diameter (M), cortical thickness (C), cortical area (CA), percent cortical area (PCA), cortical index (CI).

[d] Effect due to acidity is linear (P<.01).

Table 23. Interaction of age and calcium depletion-repletion on femur gravimetric measurements: Trial 1.

Treatment	Depleted		Repleted	
Age[a]	Young	Old	Young	Old
Femur Measurement[b]				
AD, mg	681 ± 13[c]	907 ± 24[d]	778 ± 6[c]	910 ± 11[d]
FFD, mg	399 ± 9[c]	598 ± 15[d]	492 ± 5[c]	597 ± 7[d]
FFD/AD, %	58.5 ± 0.4[c]	65.9 ± 0.5[d]	63.3 ± 0.2[c]	65.6 ± 0.2[d]
Ash, mg	235 ± 6[c]	392 ± 11[d]	300 ± 4[c]	386 ± 5[d]
Ash/AD, %	34.5 ± 0.4[c]	43.1 ± 0.6[d]	38.6 ± 0.2[c]	42.5 ± 0.2[d]
Ash/FFD, %	58.9 ± 0.4[c]	65.4 ± 0.5[d]	60.9 ± 0.2[c]	64.7 ± 0.2[d]
Volume, cc	0.4879 ± 0.0089[c]	0.5947 ± 0.0152[d]	0.5360 ± 0.0039[c]	0.6066 ± 0.0077[d]
Density, mg ash/cc	482 ± 7[c]	658 ± 9[d]	560 ± 4[c]	637 ± 5[d]
Specific Gravity, unit	1.3957 ± 0.0065[c]	1.5150 ± 0.0051[d]	1.4519 ± 0.0030[c]	1.5009 ± 0.0044[d]

[a] Values for each depleted and repleted age are means of 12 and 40 observations, respectively, ± standard error of the mean.

[b] Air dry weight (AD), fat-free dry weight (FFD).

[c,d] Means having different superscripts in the same row under the same treatment are significantly different ($P<.01$).

Table 24. Interaction of age and diet calcium on femur gravimetric measurements: Trial 1.

Age	Young				Old			
Calcium, %[a]	0.22	0.48	0.78	± S.E.[b]	0.22	0.48	0.78	± S.E.
Femur Measurement[c]								
AD, mg	753	799	793	6	893	902	938	11
FFD, mg	470	503	509[d]	5	582	594	612[d]	7
FFD/AD, %	62.5	62.9	64.4[e]	0.2	65.3	65.9	65.7	0.2
Ash, mg	282	310	314[d]	4	371	388	401[d]	5
Ash/AD, %	37.5	38.8	39.6[d]	0.2	41.6	43.0	43.0	0.2
Ash/FFD, %	60.0	61.6	61.5	0.2	63.8	65.2	65.4[d]	0.2
Volume, cc	.5235	.5487	.5421	0.0039	.6002	.5979	.6174	0.0077
Density, mg ash/cc	539	564	579[d]	4	620	649	650[d]	8
Specific Gravity, unit	1.4386	1.4555	1.4636[d]	0.0030	1.4887	1.5082	1.5096[d]	0.0044

[a] Values for 0.22, 0.48 and 0.78% calcium are means of 16, 8 and 16 observations, respectively, averaged across 3 diet protein levels and 2 diet acidity conditions with no interactions.

[b] Standard error of the mean.

[c] Air dry weight (AD), fat-free dry weight (FFD).

[d] Effect due to calcium is linear ($P<.01$).

[e] Effect due to calcium is linear ($P<.10$).

Table 25. Interaction of age and diet protein on femur gravimetric measurements: Trial 1.

Age	Young				Old			
Protein, %[a]	9	18	36	± S.E.[b]	9	18	36	± S.E.
Femur Measurement[c]								
AD, mg	758	799	798	6	911	902	913	11
FFD, mg	478	503	502	5	598	594	596	7
FFD/AD, %	63.2	62.9	63.6	0.2	65.7	65.9	65.3	0.2
Ash, mg	289	310	303	4	387	388	385	5
Ash/AD, %	38.0	38.8	39.0	0.2	42.5	43.0	42.1	0.2
Ash/FFD, %	60.2	61.6	61.3	0.2	64.7	65.2	64.6	0.2
Volume, cc	.5244	.5487	.5412	0.0039	.6056	.5979	.6121	0.0077
Density, mg ash/cc	548	564	568[d]	4	640	649	629[e]	5
Specific Gravity, unit	1.4455	1.4555	1.4566[d]	0.0030	1.5057	1.5082	1.4925[e]	0.0044

[a] Values for 9, 18 and 36% protein are means of 16, 8 and 16 observations, respectively, averaged across 3 diet Ca levels and 2 diet acidity conditions with no interactions.

[b] Standard error of the mean.

[c] Air dry weight (AD), fat-free dry weight (FFD).

[d] Effect due to protein is linear ($P<.01$).

[e] Effect due to protein is quadratic ($P<.01$).

Table 26. Interaction of age and diet acidity on femur gravimetric measurements: Trial 1.

Age	Young			Old		
Treatment[a]	Acid-Added	Natural	± S.E.[b]	Acid-Added	Natural	± S.E.
Femur Measurement[c]						
AD, mg	767[d]	789[e]	6	919	901	11
FFD, mg	482[d]	503[e]	5	600	593	7
FFD/AD, %	62.9[d]	63.7[e]	0.2	65.3	65.8	0.3
Ash, mg	294[d]	307[e]	4	386	386	5
Ash/AD, %	38.3[d]	38.9[e]	0.2	42.0[d]	42.9[e]	0.2
Ash/FFD, %	60.8[d]	61.0[e]	0.2	64.4	65.1	0.2
Volume, cc	.5307[d]	.5412[e]	0.0039	.6180	.5953	0.0077
Density, mg ash/cc	553[f]	567[g]	4	626[f]	649[g]	5
Specific Gravity, unit	1.4459[f]	1.4579[g]	0.0030	1.4881[f]	1.5138[g]	0.0044

[a] Values for each treatment are means of 20 observations, averaged across 3 diet Ca and 3 diet protein levels with no interactions.

[b] Standard error of the mean.

[c] Air dry weight (AD), fat free dry weight (FFD).

[d,e] Means having different superscripts in the same row under the same age are significantly different ($P<.05$).

[f,g] Means having different superscripts in the same row under the same age are significantly different ($P<.01$).

Table 27. Interaction of age and calcium depletion-repletion on femur mineral composition: Trial 1.

Treatment	Depleted		Repleted	
Age[a]	Young	Old	Young	Old
Mineral				
Calcium, mg	77 ± 2[b]	119 ± 3[c]	99 ± 1[b]	122 ± 2[c]
Calcium/ash, %	32.7 ± 0.4[b]	30.5 ± 0.4[c]	32.9 ± 0.2[b]	31.5 ± 0.2[c]
Magnesium, ug	1176 ± 31[b]	2570 ± 33[c]	1381 ± 20[b]	2492 ± 36[c]
Magnesium/ash, %	0.50 ± 0.01[b]	0.66 ± 0.01[c]	0.46 ± 0.01[b]	0.65 ± 0.01[c]

[a] Values for each depleted and repleted age are means of 12 and 40 observations, respectively, ± standard error of the mean.

[b,c] Means having different superscripts in the same row under the same treatment are significantly different ($P<.01$).

Table 28. Interaction of age and diet calcium on femur mineral composition: Trial 1.

Age	Young				Old			
Calcium, %[a]	0.22	0.48	0.78	± S.E.[b]	0.22	0.48	0.78	± S.E.
Mineral								
Calcium, mg	93	102	104[c]	1	117	123	125[c]	2
Calcium/ash, %	33.0	32.8	32.8	0.2	31.5	31.8	31.3	0.2
Magnesium, ug	1333	1433	1403[c]	20	2355	2577	2586[c]	36
Magnesium/ash, %	0.46	0.46	0.46	0.01	0.64	0.66	0.64	0.01

[a] Values for 0.22, 0.48 and 0.78% calcium are means of 16, 8 and 16 observations, respectively, averaged across 3 diet protein levels and 2 diet acidity conditions with no interactions.

[b] Standard error the mean.

[c] Effect due to calcium is linear ($P<.01$).

Table 29. Interaction of age and diet protein on femur mineral composition: Trial 1.

Age	Young				Old			
Protein, %[a]	9	18	36	± S.E.[b]	9	18	36	± S.E.
Mineral								
Calcium, mg	95	102	101	1	121	123	121	2
Calcium/ash, %	33.0	33.0	32.8	0.2	31.3	31.8	31.5	0.2
Magnesium, ug	1333	1433	1403	20	2453	2577	2488	36
Magnesium/ash, %	0.46	0.46	0.46	0.01	0.63	0.66	0.65	0.01

[a] Values for 9, 18 and 36% protein are means of 16, 8 and 16 observations, respectively, averaged across 3 diet Ca levels and 2 diet acidity conditions with no interactions.

[b] Standard error of the mean.

Table 30. Interaction of age and diet acidity on femur mineral composition: Trial 1.

Age	Young			Old		
Treatment[a]	Acid-Added	Natural	± S.E.[b]	Acid-Added	Natural	± S.E.
Mineral						
Calcium, mg	96	102	1	121	122	2
Calcium/ash, %	32.7	33.1	0.2	31.3	31.6	0.2
Magnesium, ug	1360	1402	20	2513	2470	36
Magnesium/ash, %	0.46	0.46	0.01	0.65	0.64	0.01

[a] Values for each treatment are means of 20 observations, averaged across 3 diet Ca and 3 diet protein levels with no interactions.

[b] Standard error of the mean.

Table 31. Interaction of age and control versus test diet on femur gravimetric measurements: Trial 2.

Treatment	Control		Test	
Age[a]	Young	Old	Young	Old
Femur Measurement[b]				
AD, mg	375 ± 24[c]	807 ± 47[d]	816 ± 9	831 ± 11
FFD, mg	150 ± 9[c]	557 ± 33[d]	439 ± 5[c]	594 ± 7[d]
FFD/AD, %	40.2 ± 0.6[c]	69.0 ± 0.6[d]	54.1 ± 0.4[c]	71.5 ± 0.3[d]
Ash, mg	74 ± 4[c]	358 ± 20[d]	257 ± 3[c]	378 ± 5[d]
Ash/AD, %	19.9 ± 0.5[c]	44.3 ± 0.6[d]	31.6 ± 0.3[c]	45.5 ± 0.1[d]
Ash/FFD, %	49.3 ± 0.6[c]	64.3 ± 0.3[d]	58.5 ± 0.4[c]	63.6 ± 0.3[d]
Volume, cc	0.2939 ± 0.0143[c]	0.5818 ± 0.0320[d]	0.6371 ± 0.0080	0.6114 ± 0.0062
Density, mg ash/cc	252 ± 10[c]	615 ± 14[d]	438 ± 7[c]	618 ± 4[d]
Specific Gravity, unit	1.2717 ± 0.0447[e]	1.3874 ± 0.0153[f]	1.3928 ± 0.0127	1.3651 ± 0.0082

[a] Values for each control and test age are means of 6 and 32 observations, respectively, ± standard error of the mean.

[b] Air dry weight (AD), fat-free dry weight (FFD).

[c,d] Means having different superscripts in the same row under the same treatment are significantly different (P<.01).

[e,f] Means having different superscripts in the same row under the same treatment are significantly different (P<.05).

Table 32. Interaction of age and diet calcium on femur gravimetric measurements: Trial 2.

Age	Young			Old		
Calcium, %[a]	0.48	0.78	± S.E.[b]	0.48	0.78	± S.E.
Femur Measurement[c]						
AD, mg	829	802	9	828	835	11
FFD, mg	435[d]	444[e]	5	588[d]	599[e]	7
FFD/AD, %	52.7[d]	55.4[e]	0.4	71.1[d]	71.9[e]	0.3
Ash, mg	252[f]	262[g]	3	373[f]	383[g]	5
Ash/AD, %	30.5[d]	32.7[e]	0.3	45.1[d]	46.0[e]	0.1
Ash/FFD, %	57.9[f]	59.0[g]	0.4	63.4[f]	63.9[g]	0.2
Volume, cc	.5976	.5765	0.0080	.6150	.6049	0.0062
Density, mg ash/cc	422[f]	454[g]	7	604[f]	633[g]	4
Specific Gravity, unit	1.3913[f]	1.3943[g]	0.0127	1.3435[f]	1.3867[f]	0.0082

[a] Values for each treatment are means of 16 observations, averaged across 4 diet acidity conditions with no interaction.

[b] Standard error of the mean.

[c] Air dry weight (AD), fat-free dry weight (FFD).

[d,e] Means having different superscripts in the same row under the same age are significantly different ($P<.05$).

[f,g] Means having different superscripts in the same row under the same age are significantly different ($P<.01$).

Table 33. Interaction of age and diet acidity on femur gravimetric measurements: Trial 2.

Age	Young					Old				
Diet Acidity, pH[a]	5.0	5.8	6.6	7.4	± S.E.[b]	5.0	5.8	6.6	7.4	± S.E.
Femur Measurement[c]										
AD, mg	799	815	822	827[d]	9	794	821	844	865[d]	11
FFD, mg	421	436	442	458[d]	5	570	589	604	613[d]	7
FFD/AD, %	52.8	53.5	54.4	55.5[e]	0.4	71.9	71.7	71.6	70.3	0.3
Ash, mg	247	254	259	270[d]	3	360	374	386	393[d]	5
Ash/AD, %	30.9	31.1	31.5	32.7[e]	0.3	45.4	45.6	45.7	45.6	0.1
Ash/FFD, %	58.7	58.3	58.6	59.0	0.4	63.2	63.5	63.9	64.1	0.2
Volume, cc	0.5834	0.5866	0.5889	0.5894[e]	0.0080	0.5861	0.6036	0.6207	0.6302[e]	0.0062
Density, mg ash/cc	423	433	440	458[d]	7	614	615	622	624[d]	4
Specific Gravity, unit	1.3757	1.3936	1.3963	1.4057[d]	0.0127	1.3538	1.3589	1.3605	1.3871[d]	0.0082

[a] Values for each treatment are means of 8 observations, averaged across 2 diet Ca levels with no interaction.

[b] Standard error of the mean.

[c] Air dry weight (AD), fat-free dry weight (FFD).

[d] Effect due to acidity is linear ($P<.01$).

[e] Effect due to acidity is linear ($P<.05$).

Table 34. Interaction of age and control versus test diet on femur mineral composition: Trial 2.

Treatment	Control		Test	
Age[a]	Young	Old	Young	Old
Mineral, % in Ash				
Calcium	38.8 ± 0.6	39.2 ± 0.3	38.1 ± 0.2[b]	39.6 ± 0.2[c]
Phosphorus	23.1 ± 0.3[b]	22.0 ± 0.3[c]	21.9 ± 0.2	22.3 ± 0.2
Magnesium	0.57 ± 0.01[d]	0.69 ± 0.02[e]	0.56 ± 0.01[d]	0.67 ± 0.01[e]
Sodium	1.31 ± 0.08	1.36 ± 0.08	1.14 ± 0.03[b]	1.35 ± 0.02[c]
Potassium	3.40 ± 0.48[d]	1.19 ± 0.13[e]	2.01 ± 0.10[d]	1.25 ± 0.06[e]
Copper	0.0069 ± 0.0019	0.0044 ± 0.007	0.0030 ± 0.0003[d]	0.0046 ± 0.0002[e]
Iron	0.0040 ± 0.0022	0.0084 ± 0.0019	0.0050 ± 0.0010[b]	0.0108 ± 0.0008[c]
Manganese	0.0187 ± 0.0025[d]	0.0044 ± 0.0012[e]	0.0085 ± 0.0009[d]	0.0044 ± 0.0004[e]
Zinc	0.037 ± 0.001[b]	0.041 ± 0.002[c]	0.040 ± 0.001[d]	0.044 ± 0.001[e]

[a] Values for each control and test age are means of 6 and 32 observations, respectively, ± standard error of the mean.

[b,c] Means having different superscripts in the same row under the same treatment are significantly different ($P<.05$).

[d,e] Means having different superscripts in the same row under the same treatment are significantly different ($P<.01$).

Table 35. Interaction of age and diet calcium on femur mineral composition: Trial 2.

Age	Young			Old		
Calcium, %[a]	0.48	0.78	± S.E.[b]	0.48	0.78	± S.E.
Mineral, % in Ash						
Calcium	38.0	38.2	0.2	39.6	39.5	0.2
Phosphorus	21.9	22.0	0.2	22.3	22.3	0.2
Magnesium	0.57	0.55	0.01	0.68	0.67	0.01
Sodium	1.14	1.14	0.03	1.35	1.34	0.02
Potassium	2.17[c]	1.84[d]	0.10	1.29[c]	1.22[d]	0.06
Copper	0.0031	0.0030	0.0003	0.0046	0.0046	0.0002
Iron	0.0045	0.0056	0.0010	0.0120	0.0097	0.0008
Manganese	0.0082	0.0088	0.0009	0.0047	0.0041	0.0004
Zinc	0.040	0.039	0.001	0.043	0.046	0.001

[a] Values for each treatment are means of 16 observations, averaged across 4 diet acidity conditions with no interaction.

[b] Standard error of the mean.

[c,d] Means having different superscripts in the same row under the same age are significantly different (P<.10).

Table 36. Interaction of age and diet acidity on femur mineral composition: Trial 2.

Age	Young					Old				
Diet Acidity, pH[a]	5.0	5.8	6.6	7.4	± S.E.[b]	5.0	5.8	6.6	7.4	± S.E.
Mineral, % in Ash										
Calcium	37.7	38.2	38.4	38.2	0.2	39.9	38.1	40.2	39.2	0.2
Phosphorus	21.9	21.7	22.0	22.0	0.2	22.5	21.9	22.5	22.2	0.2
Magnesium	0.56	0.58	0.54	0.56	0.01	0.69	0.65	0.70	0.65	0.01
Sodium	1.11	1.13	1.17	1.15	0.03	1.36	1.28	1.38	1.38	0.02
Potassium	2.00	1.93	2.00	2.10	0.10	1.27	1.16	1.26	1.32	0.06
Copper	0.0028	0.0029	0.0037	0.0029	0.0003	0.0047	0.0044	0.0047	0.0046	0.0002
Iron	0.0039	0.0038	0.0057	0.0068	0.0010	0.0101	0.0106	0.0116	0.0111	0.0008
Manganese	0.0084	0.0081	0.0088	0.0087	0.0009	0.0045	0.0043	0.0048	0.0041	0.0004
Zinc	0.039	0.041	0.037	0.042	0.001	0.043	0.043	0.045	0.045	0.001

[a] Values for each treatment are means of 8 observations, averaged across 2 diet Ca levels with no interaction.

[b] Standard error of the mean.

Table 37. Interaction of age and calcium depletion-repletion on serum composition: Trial 1.

Treatment	Depleted		Repleted	
Age[a]	Young	Old	Young	Old
Serum Constituent				
Hydroxyproline, ug/100 ml	672 ± 38[b]	352 ± 23[c]	322 ± 7[b]	240 ± 6[c]
Calcium, mg/100 ml	10.3 ± 0.1	10.4 ± 0.1	10.0 ± 0.1	10.0 ± 0.1
Magnesium, mg/100 ml	2.3 ± 0.1	2.4 ± 0.1	2.6 ± 0.1	2.6 ± 0.1
Cholesterol, mg/100 ml	61 ± 9[b]	111 ± 11[c]	106 ± 4[d]	130 ± 5[e]
Protein, g/100 ml	4.4 ± 0.1	4.8 ± 0.1	4.6 ± 0.1[b]	4.9 ± 0.1[c]

[a] Values for each depleted and repleted age are means of 12 and 40 observations, respectively, ± standard error of the mean.

[b,c] Means having different superscripts in the same row under the same treatment are significantly different (P<.01).

[d,e] Means having different superscripts in the same row under the same treatment are significantly different (P<.10).

Table 38. Interaction of age and diet calcium on serum composition: Trial 1.

Age	Young				Old			
Calcium, %[a]	0.22	0.48	0.78	± S.E.[b]	0.22	0.48	0.78	± S.E.
Serum Constituent								
Hydroxyproline, ug/100 ml	334	339	303[c]	7	260	238	222[c]	6
Calcium, mg/100 ml	9.9	10.3	10.0	0.1	9.9	10.6	9.9	0.1
Magnesium, mg/100 ml	2.7	2.6	2.6	0.1	2.7	2.6	2.6	0.1
Cholesterol, mg/100 ml	104	122	99[d]	4	132	157	113[d]	5
Protein, g/100 ml	4.6	4.5	4.6	0.1	4.9	5.0	4.9	0.1

[a] Values for 0.22, 0.48 and 0.78% calcium are means of 16, 8 and 16 observations, respectively, averaged across 3 diet protein levels and 2 diet acidity conditions with no interactions.

[b] Standard error of the mean.

[c] Effect due to calcium is linear ($P<.01$).

[d] Effect due to calcium is quadratic ($P<.10$).

Table 39. Interaction of age and diet protein on serum composition: Trial 1.

Age	Young				Old			
Protein, %[a]	9	18	36	± S.E.[b]	9	18	36	± S.E.
Serum Constituent								
Hydroxyproline, ug/100 ml	352	338	285[c]	7	259	238	223[c]	6
Calcium, mg/100 ml	10.0	10.3	9.9	0.1	9.8	10.6	10.0	0.1
Magnesium, mg/100 ml	2.7	2.6	2.7	0.1	2.6	2.6	2.6	0.1
Cholesterol, mg/100 ml	98	122	105[d]	4	119	15	127[d]	5
Protein, g/100 ml	4.6	4.5	4.6	0.1	4.9	5.0	4.9	0.1

[a] Values for 9, 18 and 36% protein are means of 16, 8 and 16 observations, respectively, averaged across 3 diet Ca levels and 2 diet acidity conditions with no interactions.

[b] Standard error of the mean.

[c] Effect due to protein is linear (P<.01).

[d] Effect due to protein is quadratic (P<.10).

Table 40. Interaction of age and diet acidity on serum composition: Trial 1.

Age	Young			Old		
Treatment[a]	Acid-Added	Natural	± S.E.[b]	Acid-Added	Natural	± S.E.
Serum Constituent						
Hydroxyproline, ug/100 ml	341[c]	303[d]	7	259[c]	222[d]	6
Calcium, mg/100 ml	9.7[e]	10.3[f]	0.1	9.8[e]	10.3[f]	0.1
Magnesium, mg/100 ml	2.8[e]	2.5[f]	0.1	2.7[e]	2.5[f]	0.1
Cholesterol, mg/100 ml	103[g]	109[h]	4	120[g]	139[h]	5
Protein, g/100 ml	4.4[e]	4.7[f]	0.1	4.8[e]	5.0[f]	0.1

[a] Values for each treatment are means of 20 observations, averaged across 3 diet Ca and 3 diet protein levels with no interactions.

[b] Standard error of the mean.

[c,d] Means having different superscripts in the same row under the same age are significantly different (P<.01).

[e,f] Means having different superscripts in the same row under the same age are significantly different (P<.05).

[g,h] Means having different superscripts in the same row under the same age are significantly different (P<.10).

Table 41. Interaction of age and control versus test diet on serum composition: Trial 2.

Treatment	Control		Test	
Age[a]	Young	Old	Young	Old
Serum Constituent				
Hydroxyproline, ug/100 ml	1131 ± 29[b]	450 ± 42[c]	636 ± 8[b]	420 ± 7[c]
Calcium, mg/100 ml	7.8 ± 0.1	7.8 ± 0.2	8.4 ± 0.1	8.3 ± 0.1
Magnesium, mg/100 ml	2.4 ± 0.1	2.4 ± 0.3	3.5 ± 0.1[d]	3.0 ± 0.1[e]
Cholesterol, mg/100 ml	54 ± 2	62 ± 3	52 ± 1[b]	59 ± 1[c]

[a] Values for each control and test age are means of 6 and 32 observations, respectively, ± standard error of the mean.

[b,c] Means having different superscripts in the same row under the same treatment are significantly different (P<.01).

[d,e] Means having different superscripts in the same row under the same treatment are significantly different (P<.05).

Table 42. Interaction of age and diet calcium on serum composition: Trial 2.

Age	Young			Old		
Calcium, %[a]	0.48	0.78	± S.E.[b]	0.48	0.78	± S.E.
Serum Constituent						
Hydroxyproline, ug/100 ml	644[c]	627[d]	8	428[c]	412[d]	7
Calcium, mg/100 ml	8.5	8.4	0.1	8.2	8.4	0.1
Magnesium, mg/100 ml	3.6	3.4	0.1	3.0	3.0	0.1
Cholesterol, mg/100 ml	54[c]	49[d]	1	61[c]	56[d]	1

[a] Values for each treatment are means of 16 observations, averaged across 4 diet acidity conditions with no interaction.

[b] Standard error of the mean.

[c,d] Means having different superscripts in the same row under the same age are significantly different ($P<.05$).

Table 43. Interaction of age and diet acidity on serum composition: Trial 2.

Age	Young					Old				
Diet Acidity, pH[a]	5.0	5.8	6.6	7.4	± S.E.[b]	5.0	5.8	6.6	7.4	± S.E.
Serum Constituent										
Hydroxyproline, ug/100 ml	669	644	623	606[c]	8	446	426	411	396[c]	7
Calcium, mg/100 ml	8.5	8.5	8.4	8.4	0.1	8.4	8.2	8.5	8.1	0.1
Magnesium, mg/100 ml	3.6	3.4	3.6	3.3	0.1	3.1	3.0	3.1	2.8	0.1
Cholesterol, mg/100 ml	51	53	56	46[d]	1	56	62	65	52[d]	1

[a] Values for each treatment are means of 8 observations, averaged across 2 diet Ca levels with no interaction.

[b] Standard error of the mean.

[c] Effect due to acidity is linear ($P<.01$).

[d] Effect due to acidity is quadratic ($P<.01$).

Table 44. Interaction of age and calcium depletion-repletion on soft tissue mineralization: Trial 1.

Treatment	Depleted		Repleted	
Age[a]	Young	Old	Young	Old
Tissue[b]				
Heart				
Calcium, ppm	297 ± 13	268 ± 12	382 ± 9[c]	326 ± 6[d]
Magnesium, ppm	857 ± 14	835 ± 8	829 ± 5	814 ± 6
Kidney				
Calcium, ppm	17,648 ± 2303[c]	228 ± 7[d]	11,395 ± 895[c]	322 ± 6[d]
Magnesium, ppm	1,015 ± 51[c]	654 ± 12[d]	778 ± 14[c]	582 ± 9[d]

[a] Values for each depleted and repleted age are means of 12 and 40 observations, respectively, ± standard error of the mean.

[b] Values are expressed on a fat-free dry basis.

[c,d] Means having different superscripts in the same row under the same treatment are significantly different ($P<.01$).

Table 45. Interaction of age and diet calcium on soft tissue mineralization: Trial 1.

Age	Young				Old			
Calcium, %[a]	0.22	0.48	0.78	± S.E.[b]	0.22	0.48	0.78	± S.E.
Tissue[c]								
Heart								
Calcium, ppm	332	387	430[d]	9	301	332	348[c]	6
Magnesium, ppm	840	831	818	5	828	811	803	6
Kidney								
Calcium, ppm	16,590	11,860	5,968[d]	895	294	332	346[d]	6
Magnesium, ppm	865	797	683[d]	14	611	580	555[d]	9

a Values for 0.22, 0.48 and 0.78% calcium are means of 16, 8 and 16 observations, respectively, averaged across 3 diet protein levels and 2 diet acidity conditions with no interaction.

b Standard error of the mean.

c Values are expressed on a fat-free dry basis.

d Effect due to calcium is linear ($P<.01$).

Table 46. Interaction of age and diet protein on soft tissue mineralization: Trial 1.

Age	Young				Old			
Protein, %[a]	9	18	36	± S.E.[b]	9	18	36	± S.E.
Tissue[c]								
Heart								
Calcium, ppm	377	387	386	9	322	332	326	6
Magnesium, ppm	834	831	822	5	821	811	810	6
Kidney								
Calcium, ppm	10,761	11,860	11,797	895	313	332	327	6
Magnesium, ppm	763	797	785	14	597	580	569	9

[a] Values for 9, 18 and 36% protein are means of 16, 8 and 16 observations, respectively, averaged across 3 diet Ca levels and 2 diet acidity conditions with no interactions.

[b] Standard error of the mean.

[c] Values are expressed on a fat-free dry basis.

Table 47. Interaction of age and diet acidity on soft tissue mineralization: Trial 1.

Age	Young			Old		
Treatment[a]	Acid-Added	Natural	± S.E.[b]	Acid-Added	Natural	± S.E.
Tissue[b]						
Heart						
Calcium, ppm	389[d]	375[e]	9	336[d]	315[e]	6
Magnesium, ppm	818[f]	840[g]	5	806[f]	822[g]	6
Kidney						
Calcium, ppm	12,688[f]	10,122[g]	395	332[f]	312[g]	6
Magnesium, ppm	799[f]	758[g]	14	565[f]	599[g]	9

[a] Values for each treatment are means of 20 observations, averaged across 3 diet Ca and 3 diet protein levels with no interactions.

[b] Standard error of the mean.

[c] Values are expressed on a fat-free dry basis.

[d,e] Means having different superscripts in the same row under the same age are significantly different ($P<.10$).

[f,g] Means having different superscripts in the same row under the same age are significantly different ($P<.05$).

Table 48. Interaction of age and control versus test diet on soft tissue mineralization: Trial 2.

Treatment	Control		Test	
Age[a]	Young	Old	Young	Old
Tissue[b]				
Heart				
Calcium, ppm	215 ± 10[c]	141 ± 7[d]	197 ± 6	210 ± 5
Magnesium, ppm	669 ± 24	612 ± 35	813 ± 10[c]	886 ± 8[d]
Kidney				
Calcium, ppm	473 ± 34[e]	392 ± 27[f]	34,883 ± 4978[c]	400 ± 7[d]
Magnesium, ppm	931 ± 28[c]	639 ± 52[d]	1,264 ± 83[c]	713 ± 14[d]

[a] Values for each control and test age are means of 6 and 32 observations, respectively, ± standard error of the mean.

[b] Values are expressed on a fat-free dry basis.

[c,d] Means having different superscripts in the same row under the same treatment are significantly different ($P<.01$).

[e,f] Means having different superscripts in the same row under the same treatment are significantly different ($P<.05$).

Table 49. Interaction of age and diet calcium on soft tissue mineralization: Trial 2.

Age	Young			Old		
Calcium, %[a]	0.48	0.78	±S.E.[b]	0.48	0.78	± S.E.
Tissue[c]						
Heart						
Calcium, ppm	187[d]	207[e]	6	204[d]	215[e]	5
Magnesium, ppm	828[d]	799[e]	10	899[d]	873[e]	8
Kidney						
Calcium, ppm	44,209[f]	25,559[g]	4978	396	404	7
Magnesium, ppm	1,447[f]	1,082[g]	83	727	700	14

[a] Values for each treatment are means of 16 observations, averaged across 4 diet acidity conditions with no interaction.

[b] Standard error of the mean.

[c] Values are expressed on a fat-free dry basis.

[d,e] Means having different superscripts in the same row under the same age are significantly different (P<.05).

[f,g] Means having different superscripts in the same row under the same age are significantly different (P<.01).

Table 50. Interaction of age and diet acidity on soft tissue mineralization: Trial 2.

Age[a]	Young					Old				
Diet Acidity, pH[a]	5.0	5.8	6.6	7.4	± S.E.[b]	5.0	5.8	6.6	7.4	± S.E.
Tissue[c]										
Heart										
Calcium, ppm	126	209	197	172[d]	4	91	216	199	193[d]	5
Magnesium, ppm	770	913	827	944[d]	10	809	880	896	911[d]	8
Kidney										
Calcium, ppm	11,320	29,905	61,467	36,844[e]	4979	432	415	390	365[d]	7
Magnesium, ppm	934	1,159	1,375	1,290[e]	93	609	705	735	746[d]	14

[a] Values for each treatment are means of 8 observations, averaged across 7 diet Ca levels with no interaction.

[b] Standard error of the mean.

[c] Values are expressed on a fat-free dry basis.

[d] Effect due to acidity is linear (P<.01).

[e] Effect due to acidity is quadratic (P<.05).

Figure 1.

A. Nephrocalcinosis in a young rat following a 7 week period of Ca depletion with a Ca-deficient (0.16%), low Mg (0.041%) diet (Table 44). This rat (#6C) had 18,178 ppm kidney Ca by analysis. Von Kossa, X14.

B. Normal kidney of an old rat following a 7 week period of Ca depletion with a Ca-deficient (0.16%), low Mg (0.041%) diet (Table 44). This rat (#61C) had 266 ppm kidney Ca by analysis. Von Kossa, X14.

C. Moderate nephrocalcinosis in a young Ca-depleted rat fed an acid-added diet (Table 47). This rat (#A9) had 17,345 ppm kidney Ca by analysis. Von Kossa, X14.

D. Slight nephrocalcinosis in a young Ca-depleted rat fed a non acid-added diet (Table 47). This rat (#A12) had 3485 ppm kidney Ca by analysis. Von Kossa, X14.

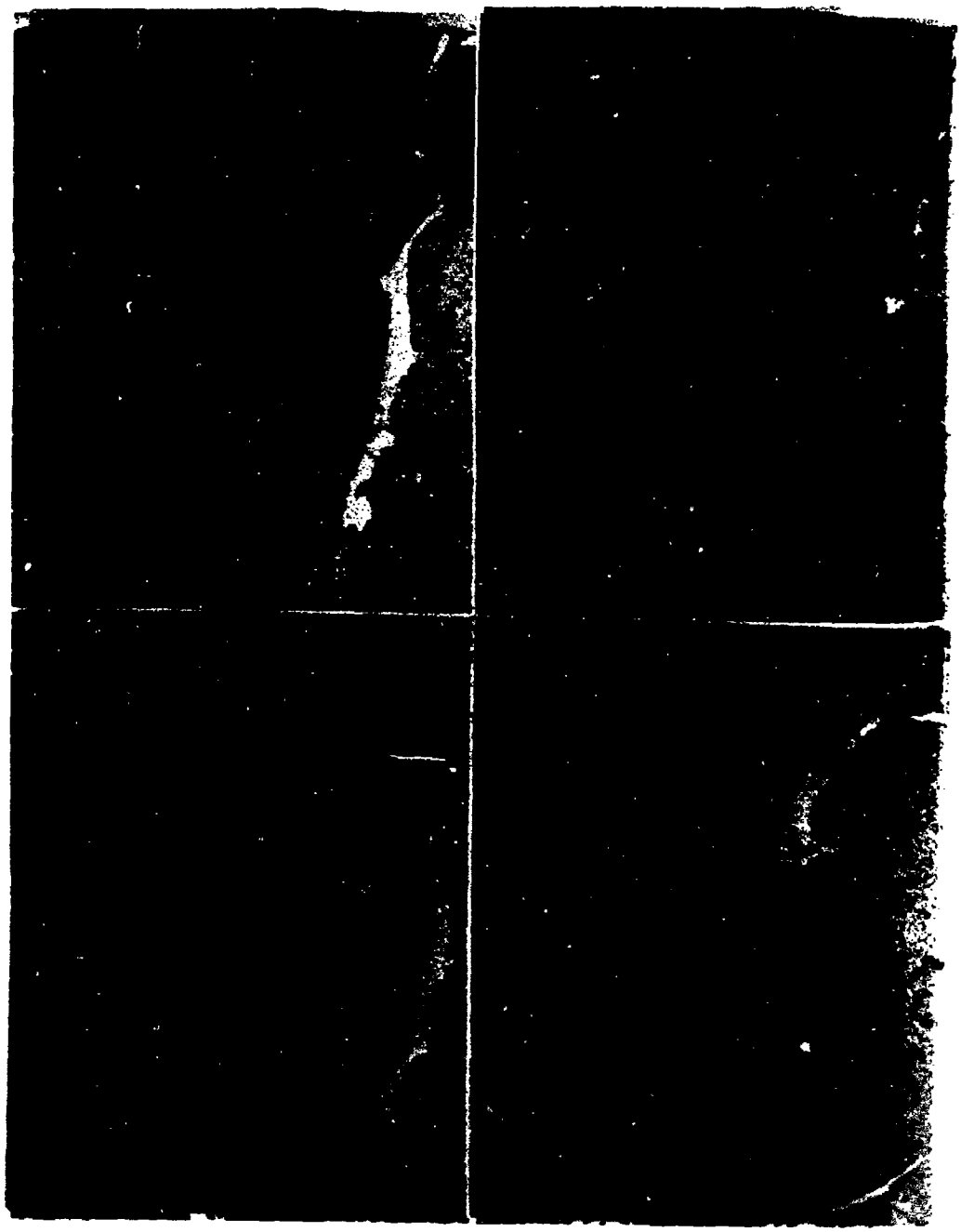


Figure 2.

A. Slight nephrocalcinosis in a young Ca-depleted rat fed a 0.78% Ca diet (Table 45). This rat (#C39) had 3536 ppm kidney Ca by analysis. Von Kossa, X14.

B. Moderate nephrocalcinosis in a young Ca-depleted rat fed a 0.22% Ca diet (Table 45). This rat (#A2) had 17,240 ppm kidney Ca by analysis. Von Kossa, X14.

C. Slight nephrocalcinosis in a young non-depleted rat fed a 0.78% Ca diet (Table 49). This rat (#L34) had 4379 ppm kidney Ca by analysis. Von Kossa, X14.

D. Moderate nephrocalcinosis in a young non-depleted rat fed a 0.48% Ca diet (Table 49). This rat (#K22) had 21,297 ppm kidney Ca by analysis. Von Kossa, X14.

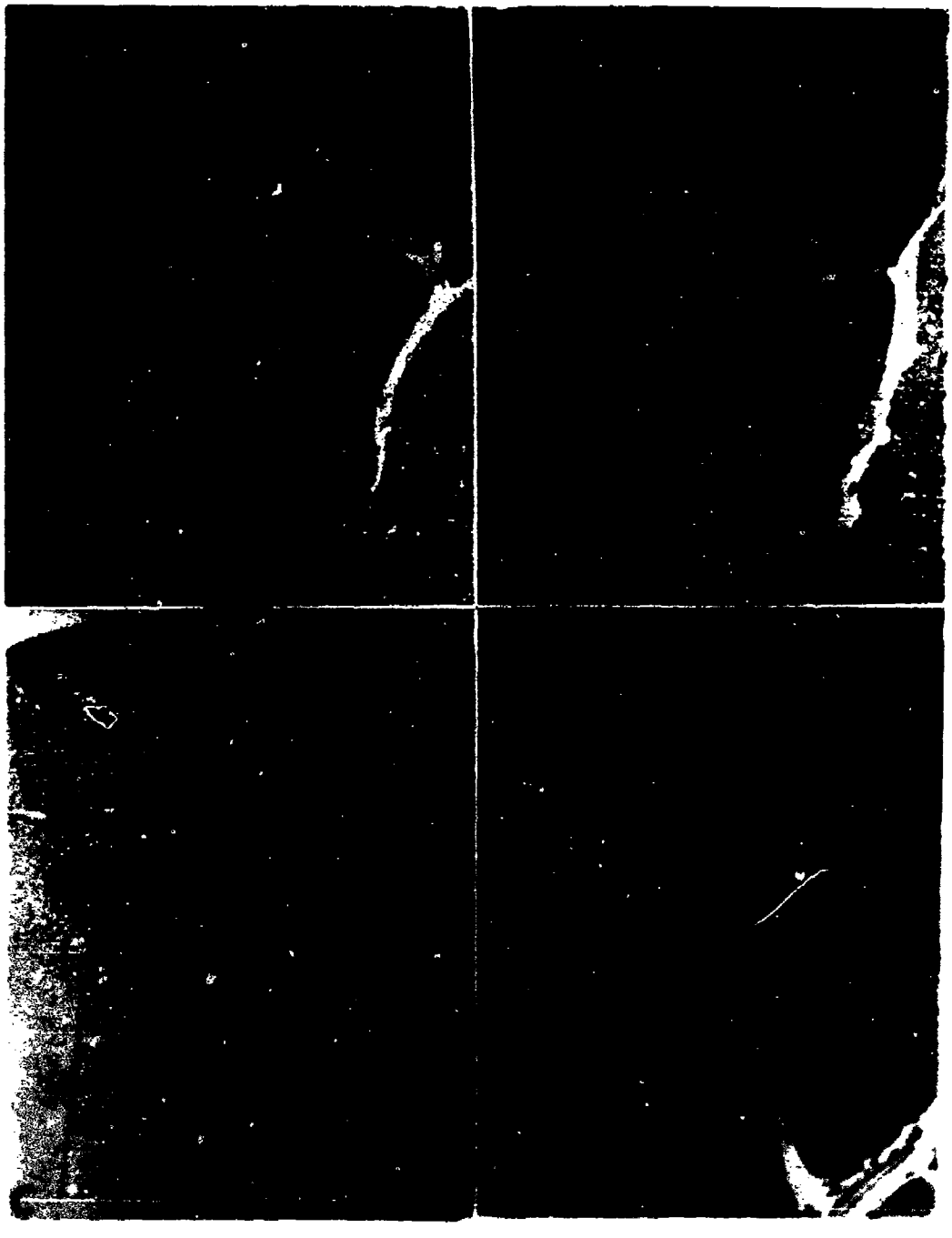


Figure 3.

A. Slight nephrocalcinosis in a young non-depleted rat fed a pH 5.0 diet (Table 50). This rat (#L37) had 2595 ppm kidney Ca by analysis. Von Kossa, X14.

B. Moderate nephrocalcinosis in a young non-depleted rat fed a pH 5.8 diet (Table 50). This rat (#L39) had 21,276 ppm kidney Ca by analysis. Von Kossa, X14.

C. Very severe nephrocalcinosis in a young non-depleted rat fed a pH 6.6 diet (Table 50). This rat (#L33) had 93,444 ppm kidney Ca by analysis. Von Kossa, X14.

D. Severe nephrocalcinosis in a young non-depleted rat fed a pH 7.4 diet (Table 50). This rat (#L35) had 49,803 ppm kidney Ca by analysis. Von Kossa, X14.

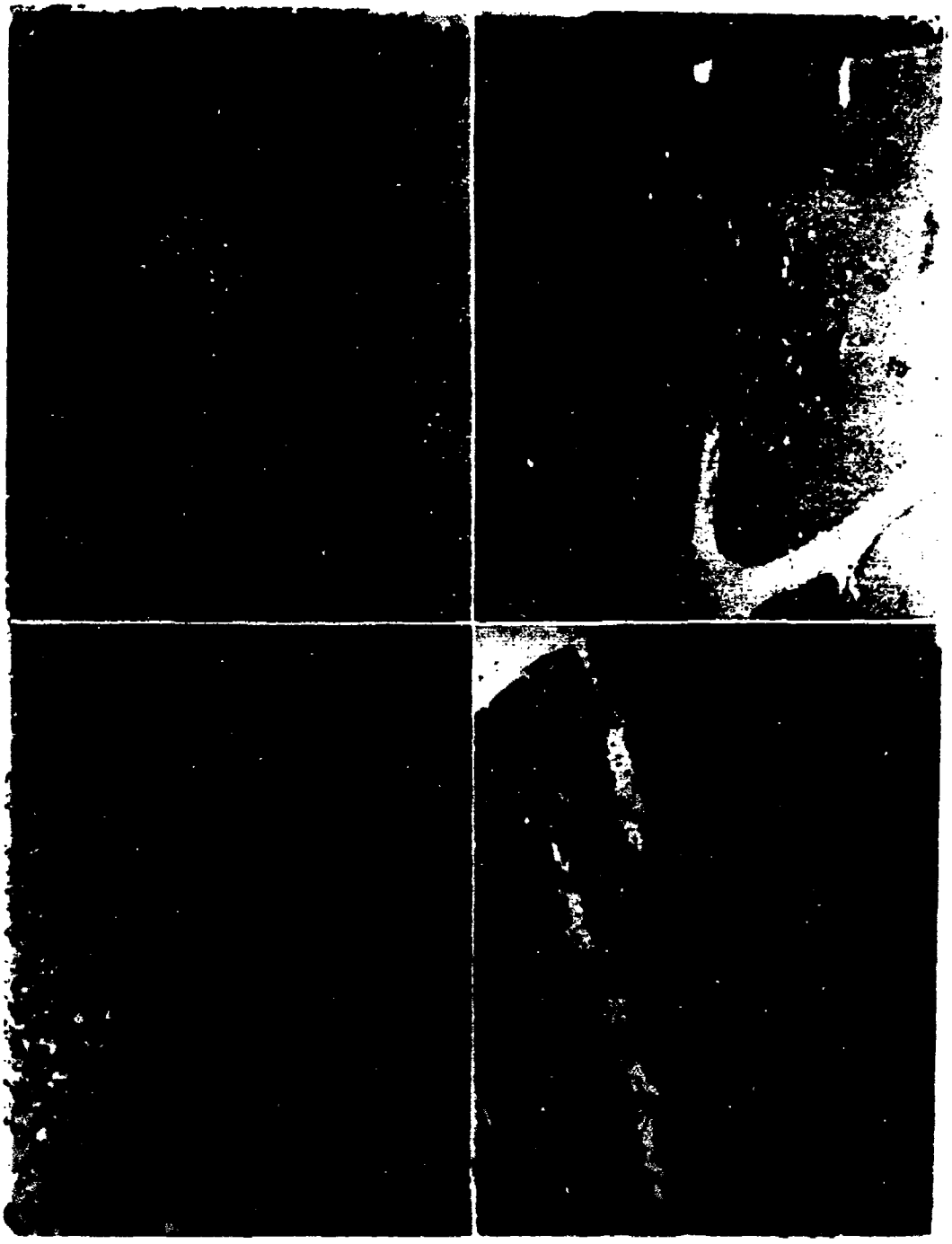


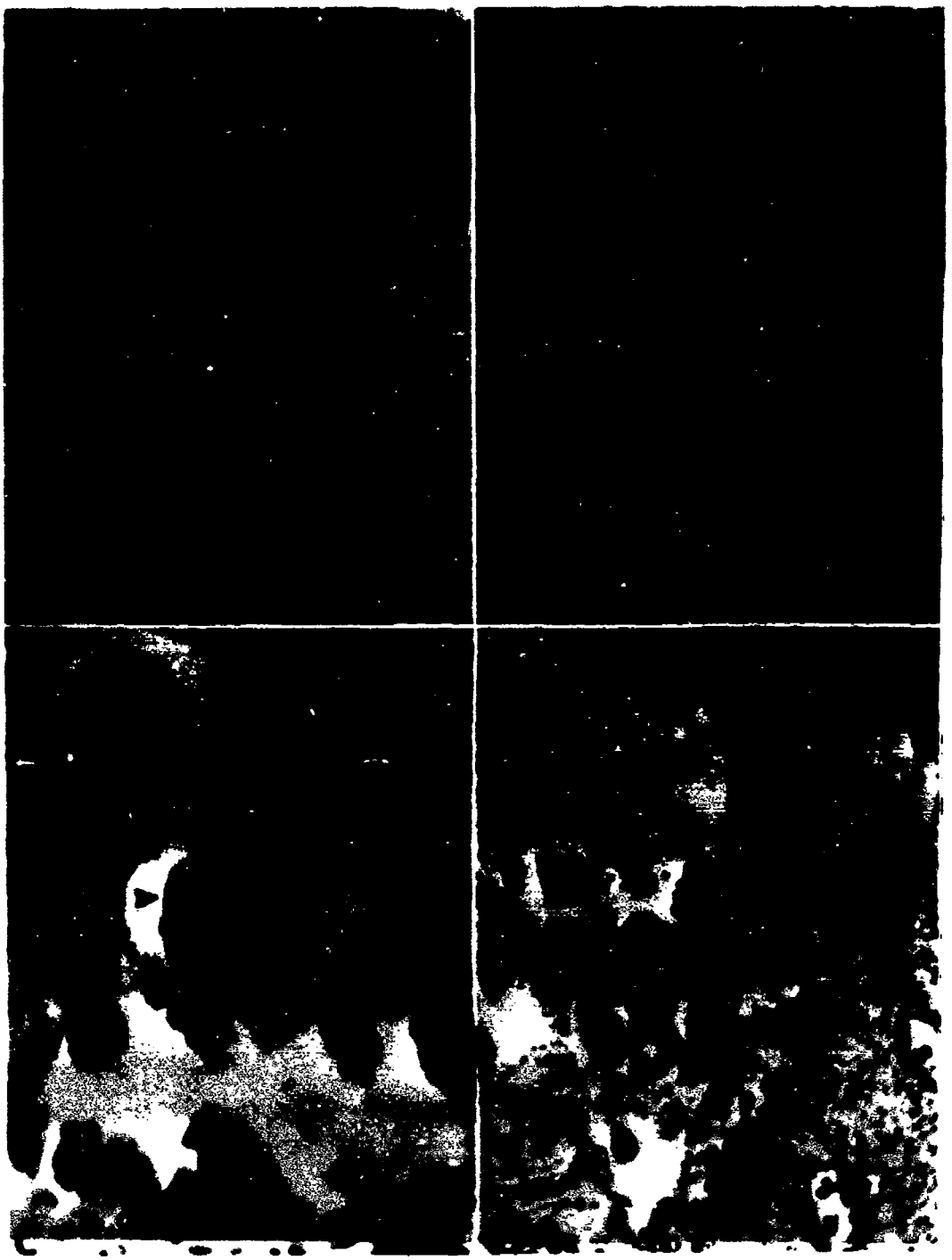
Figure 4.

A. Low diet Mg-induced nephrocalcinosis is confined to the medullary region of the kidney. Von Kossa, X14.

B. There is no glomerular involvement in low diet Mg-induced nephrocalcinosis. Von Kossa, X180.

C. Low diet Mg-induced nephrocalcinosis begins in the basement membrane (arrows) and later involves the the tubular epithelial cells. Von Kossa, X450.

D. Low diet Mg-induced nephrocalcinosis eventually leads to complete disintegration of the involved kidney tubules. Von Kossa, X180.



V. DISCUSSION

A. Dietary Effects on Osteopenia

1. Effects of Calcium

It has been stated that "osteoporosis" can be produced and cured in rats by low and high Ca diets (111). It has certainly been shown that diets containing levels of Ca below NRC recommendations result in increased bone resorption and decreased bone deposition in rats (67). This effect has been shown to result in decreased bone ash and "osteoporosis" (312). By the same token, ingesting of diet Ca levels above NRC recommendations has been reported to increase skeletal calcification (105). However, 0.48% diet Ca has been reported as the level necessary for maximal mineralization of the rat skeleton (37). Another study shows cortical thickness of rat bones increasing with increasing diet Ca up to 0.36% (33). Similarly, a curvilinear increase in bone density in humans has been shown with increasing diet Ca up to 0.50% (308). Still another study with rats (in 2 separate trials) showed femur Ca increasing with increased diet Ca between 0.32 to 0.64% and between 0.18 to 0.69% with no further increase in femur Ca above 0.64 or 0.69% diet Ca (266). It would seem, therefore, that with increasing diet Ca, a point is reached where the amount of Ca available for bone mineralization is at the saturation level. The data in the present investigation are in agreement with the above findings.

In Trial 1, the repleted young rats had greater bone

deposition at both the subperiosteal and endosteal surfaces, as shown radiographically (Table 16 and Figure 5A) and confirmed histopathologically (Figure 6), compared to the Ca-depleted (0.16%) controls. Furthermore, the repleted young rats had greater femur Ca and Mg (Table 27), ash, density and specific gravity (Table 23) compared to the Ca-depleted controls. The greater femur density of the repleted young rats, compared to the Ca-depleted controls, was also confirmed by histopathologic examination (Figure 6). On transverse sections, the larger resorption cavities and almost non-existent subperiosteal lamellar bone layer of the thin femur cortex of the Ca-depleted young rat were evident (Figure 6A). Conversely, Ca repletion was shown to result in a much wider cortex resulting from grossly thickened lamellar bone layers at both surfaces, in addition to an abundance of cementing lines and retention of chondroid core (Figure 6B). On longitudinal sections, Ca depletion of the young rat was shown to produce thin trabeculae (Figure 6C), while repletion of the young rat resulted in thick trabecular bone with a considerable amount of chondroid core retained in the diaphyseal secondary spongiosa (Figure 6D).

The conclusion, therefore, is that Ca depletion does produce osteopenia ("osteoporosis") in the young rat, through the mechanisms of increased bone resporption and decreased bone deposition. In addition, Ca repletion of the young rat overcomes this "osteoporotic" condition through increased bone deposition and decreased resorption. This conclusion

is further supported by the lower serum hydroxyproline (HP) levels of the repleted young rats (Table 37) and is in agreement with other researchers who suggest that Ca supplementation can overcome "osteoporosis" in man (64), dogs (170) and rats (267).

Ca depletion-repletion showed no significant effects on old rat femurs, either radiographically (Table 16), gravimetrically (Table 23), chemically (Table 27) or histopathologically. The old rats seemed to be more refractory to changes in diet Ca. This finding is in agreement with Romasz (266) who also showed that the bone of rats becomes less susceptible to diet Ca manipulation with age. This effect is probably due to a smaller exchangeable Ca pool in the bone of older animals (180,181,222).

In Trial 2, young rats at the end of the 7 week experimental period had increased femur radiographic (Table 20 and Figure 5B) and gravimetric (Table 31) measurements, as well as reduced serum HP (Table 41), compared to the randomly selected initial controls. But these differences can be contributed to normal bone growth and aging rather than a function of "osteoporotic" resorption. This was ably demonstrated by histopathological examination (Figure 7). In transverse sections, the femur cortex of the initial control rat (although thin and porous) had a thick subperiosteal lamellar layer, numerous cementing lines and a considerable amount of retained chondroid core indicating normal bone deposition and mineralization (Figure 7A). At the end of

the experimental period, the femur cortex was wider as a result of growth, but no difference existed in lamellar bone, cementing lines or chondroid core retention (Figure 7B). Similarly in longitudinal sections, the experimental feeding period resulted in a more dense secondary spongiosia with thicker trabeculae (Figure 7D) than was present in the younger initial control (Figure 7C). However, the relative amounts of retained chondroid core in the femur secondary spongiosa of the 2 groups is basically the same indicating no "osteoporotic" condition present in Trial 2.

In Trial 1, increasing the repletion diet Ca level from 0.22 to 0.78% improved the degree of recovery from "osteoporosis" caused by the 6 week Ca depletion period. This was shown by an increase in the radiographic measurements T, M and CA (Table 17 and Figure 8A). Cortical thickness, PCA and CI were reduced with increased diet Ca due to an increased stimulation in rate of bone turnover at the endosteal surface. This finding is consistent with previous reports of rats recovering from Ca deficiency (126). Gravimetric measurements also showed the beneficial effect of increasing the Ca level of the repletion diets fed to young rats (Table 24). This latter effect is the result of increased bone deposition in relation to resorption with increased diet Ca, as indicated by decreased serum HP levels (Table 38) and confirmed by histopathologic examination (Figure 9). In transverse sections of young Trial 1 rat femurs, it was shown that the 0.78% Ca

diet resulted in more numerous cementing lines and relatively more retained chondroid core (Figure 9B) than did the 0.22% Ca diet (Figure 9A). In longitudinal sections, it was evident that the 0.78% Ca diet resulted in thicker trabeculae with considerably more chondroid core (Figure 9D) than did the 0.22% Ca diet (Figure 9C).

The old rats in Trial 1 proved to be refractory to radiographic changes with increased Ca level in the repletion diet (Table 17). However, they were gravimetrically similar to the young rats (Table 24), as well as showing the same response in serum HP with increased diet Ca (Table 38). This finding should therefore give hope that, despite the refractoriness of mature bone, a diligent program of Ca supplementation might overcome the effects of "osteoporosis" in older individuals as suggested by other researchers (170,173, 176). Furthermore, serum HP may be a useful tool for the early diagnosis of "osteoporosis" in the aged when the other clinical signs are still negative.

In Trial 2, the normally mineralized femurs of both young and old rats were refractory to radiographic changes with increased diet Ca (Table 21). Nevertheless, increasing diet Ca from 0.48 to 0.78% did produce gravimetric changes in the femurs of both age groups (Table 32) similar to the changes in Trial 1. Serum HP changes in Trial 2 (Table 42) were also identical to Trial 1. However, histopathologically, very little (if any) difference can be seen in femurs from the 2 diet Ca regimens of Trial 2 (Figure 10) since at both

0.48 and 0.78% Ca, the femurs of non Ca-depleted rats were well mineralized.

With one exception, diet Ca level had no effect on femur mineral composition of either age group of rats in both trials when those minerals were expressed as percent in ash. That one exception was K in Trial 2. With increased diet Ca, femur K percent in ash was significantly decreased in both young and old rats (Table 35). Potassium is the major intracellular cation as well as the major cation present in the extracellular fluid of bone (46,184). Decreased K percent in ash with increased diet Ca may represent a cation exchange. It may also represent a decrease in the extracellular fluid content and/or matrix of bone with increased mineralization. Otherwise, it can be seen from this study that, chemically, bone is unaffected by diet treatment. Age, on the other hand, created some significant differences in femur mineral composition (Table 34).

2. Effects of Protein

Optimal skeletal development requires optimal nutrition. The optimum refers to amounts of and balance between nutrients and varies with species, physiological state and age. Since retarded growth is a common denominator of imbalances and deficiencies, maximal growth is often considered a criterion of optimal nutrition. There is evidence, however, that optimal nutrition in terms of growth may be overnutrition in terms of skeletal development (131). Overnutrition is an excessive intake of a complete diet or of a

specific nutrient. Clinical and experimental studies have indicated negative effects on skeletal development by over-nutrition and too rapid growth in both children (79) and rats (269). Although some researchers have shown that increased but not excessive diet protein fed to rats results in increased femur ash, Ca and density (87), other researchers have demonstrated that unrestricted amounts of protein fed to rats results in decreased cortical thickness (269). The present study confirmed the above findings.

In the young rats of Trial 1, increased diet protein resulted in decreased bone resorption as confirmed by decreased serum HP levels (Table 39), and increased femur density and specific gravity as shown by gravimetric measurements (Table 25). Both findings were confirmed by histopathologic examination (Figure 11). Transverse sections of femurs of young rats showed a predominance of "arrested resorption", cementing lines in the cortex of rats fed 36% protein (Figure 11B) compared to the absence of such lines in the cortex of rats fed 9% protein (Figure 11A). Retained chondroid core in the cortex of young rats fed 36% protein (Figure 11B) was another indication of decreased resorption and therefore increased femur density with increased diet protein. Similarly, longitudinal sections of femurs of young rats showed thin trabeculae with an absence of chondroid core in the secondary spongiosa of the diaphysis of rats fed 9% protein (Figure 11C), compared to the thick trabeculae with abundant retained chondroid core of rats fed 36% protein (Figure 11D). Besides

the increased femur density in the young rats of Trial 1, increased diet protein also resulted in increased bone turnover and increased total cortical area as demonstrated radiographically (Table 18). The increased bone turnover was characterized by increased subperiosteal deposition and increased endosteal resorption (Figure 8B). The increased subperiosteal lamellar thickening was also confirmed histopathologically (Figure 11B). However, despite the increased bone turnover, CA and density with increased diet protein in the young rat, C, PCA and CI were reduced (Table 18 and Figure 8B). The conclusion, therefore, in the young growing rat is that maximal skeletal growth rate stimulated by high diet protein may be incompatible with optimal skeletal characteristics.

In the old rats of Trial 1, increased diet protein from 9 to 18% also resulted in decreased bone resorption as confirmed by decreased serum HP levels (Table 39), and increased femur density and specific gravity as shown by gravimetric measurements (Table 25). The effects of increased diet protein on decreased bone resorption and increased bone density was probably mediated through hypercalcitoninism, as has been shown in dogs (131). These effects were shown by histopathologic section (Figure 12). Transverse sections of femurs of old rats showed an absence of cementing lines or retained chondroid core in rats fed 9% diet protein (Figure 12A), and a predominance of cementing lines, retained chondroid core and a thick subperiosteal lamellar region in

rats fed 18% diet protein (Figure 12B). Similarly, in longitudinal sections, the 9% protein fed rat had thin trabecular bone with no retained chondroid core (Figure 12D), while the 18% protein fed rat had thick trabecular bone with abundant chondroid core (Figure 12E), indicating decreased resorption and increased density.

The excessive increase in diet protein from 18 to 36% resulted in osteopenia in the old rats of Trial 1 as demonstrated by decreases in femur density and specific gravity (Table 25). This finding is consistent with a recent report by Beecher and Coupain (27) which suggested that "osteoporotic" like conditions are induced by feeding high diet protein to rats during adulthood. Decreased femur density, in itself, might suggest increased bone resorption as a result of hyperparathyroidism. Nutritional secondary hyperparathyroidism could occur with excessive diet protein as a result of the high P content of protein. However, the diet P level was maintained at a constant level (0.44%) in Trial 1 to eliminate the Ca:P variable. Furthermore, Beecher and Coupain showed that high diet protein-induced "osteoporosis" occurs independent of diet P level (27). Increased bone resorption could occur with excessive diet protein as a result of the acid-ash nature of protein. The present study has shown the osteopenic effect of increased diet acidity (Tables 19, 22, 26, 33). The excessive diet protein-induced osteopenia in old rats in Trial 1 cannot be mediated through increased bone resorption, however, because

serum HP levels were further reduced in the 36% protein fed old rats (Table 39). Instead, the osteopenia seen here is probably a further manifestation of hypercalcitoninism which would be in accord with the findings in dogs with hypercalitoninism produced by protein overnutrition (131). The reduced femur density and serum HP levels in the 36% protein fed old rats reflect decreased bone deposition and retarded bone remodeling as shown by histopathologic section (Figure 12). The femur transverse section of the 36% protein fed old rat showed a thick, well established endosteal lamellar bone layer indicating retarded remodeling (Figure 12C), a result of hypercalcitoninism. In the longitudinal section, the 36% protein fed old rat had sparse, thin and irregular trabecular bone (Figure 12F). This latter effect of excessive diet protein may be the result of hypercalcitoninism inhibiting normal cartilage (and therefore bone) formation, as shown in dogs (131), and/or a direct action of acidosis in retarding bone deposition. It is therefore clear that, in the old rat, excess diet protein produces undesirable skeletal characteristics that are not detectable by radiogrammetry (Table 18) or mineral analyses (Table 29).

3. Effects of Acidity

It has often been suggested that bone acts to buffer hydrogen ions and in doing so releases Ca ions (61,282). Leamann et al. (192) offered further evidence for the participation of bone mineral in the defense against chronic metabolic acidosis. They reported that "as the acid load is

initiated, extracellular and intracellular buffer systems are titrated. As acid retention continues, intracellular and bone buffers and, finally bone mineral alone, appear to provide the additional buffer reserves." This action of bone as a buffer has further been shown to result in bone loss. For example, chronic ingestion of NH_4Cl in rats resulted in significant loss of bone tissue, including both the organic and inorganic phases of bone, with no change in serum Ca (22). This bone loss was due to increased resorption. Conversely, chronic ingestion of alkali resulted in prevention of osteopenia, due to increased bone formation (22). Another study also showed that the excessive administration of NH_4Cl to rats caused the development of "osteoporosis" (19). The "osteoporosis" was due to loss of bone substance and bone mineral associated with increased bone resorption. The bone loss in the latter study was indicated by a decrease in bone length, volume, density, fat-free dry weight, ash and Ca. Histopathologic examination in that study showed that these bones were indeed "osteoporotic", with less and thinner trabeculae, and with no abnormal osteoid seams or cementing lines.

The data of the present study reinforces the validity of these findings. In both Ca-depleted and non-depleted young rats, increased diet acidity resulted in reduced subperiosteal deposition and increased endosteal turnover (Tables 19, 22 and Figure 13), both indicative of increased bone resorption. Retarded subperiosteal deposition in both

Ca-depleted and non-depleted old rats with increased diet acidity also indicate the demand on bone mineral as a buffer (Tables 19 and 22). Increased diet acidity also resulted in decreased femur density and specific gravity in both age groups of Ca-depleted (Table 27) and non-depleted (Table 33) rats. This "osteoporotic" effect of diet acidity was confirmed by histopathologic examination of femurs of young rats (Figures 14-16). Transverse sections of Trial 1 young femurs showed that diet acid addition resulted in a thin lamellar layer at both bone surfaces, and a lack of cementing lines and retained chondroid core (Figure 14A). Conversely, in the young Trial 1 rat the natural diet resulted in thickened lamellar bone at both surfaces, as well as an abundance of cementing lines and considerable amounts of retained chondroid core, indicating decreased resorption (Figure 14B). Longitudinal sections of Trial 1 young rat femurs showed that diet acid addition results in thin sparse trabeculae in the secondary spongiosa of the diaphysis (Figure 14C), while the natural diet produces thick trabeculae with considerable amounts of retained chondroid core (Figure 14D). Transverse sections of Trial 2 young rat femurs showed that the pH 5.0 diet resulted in numerous resorption cavities (Figures 15A), while the pH 5.8 diet resulted in numerous resorption cavities and an occasional cementing line indicating infrequent periods of decreased resorption (Figure 15B). The pH 6.6 diet resulted in a slightly thickened lamellar layer at both bone surfaces, plus

numerous cementing lines and some retained chondroid core (Figure 15C). The pH 7.4 diet resulted in a greatly thickened lamellar layer at both surfaces, extensive cementing lines and an abundance of chondroid core (Figure 15D). Longitudinal sections of Trial 2 young rat femurs showed that the trabeculae of the diaphyseal secondary spongiosa are thin and sparse with the PH 5.0 diet (Figure 16A), and are thicker with relatively small amounts of chondroid core with the pH 5.8 diet (Figure 16B). The pH 6.6 diet resulted in an even greater thickening of the trabeculae with a greater amount of retained chondroid core (Figure 16C), while the femur diaphysis of the young rat fed the pH 7.4 diet was extensively laced with trabecular bone which contained an abundant amount of retained chondroid core (Figure 16D).

The "osteoporotic" effect of diet acidity through a general mechanism of increased bone resorption was further supported by an increase in serum HP with increased diet acidity in young and old rats in both trials (Tables 40 and 43). Increased serum HP levels have been shown to be a reliable indicator of increased bone catabolism in rats (266).

The specific "osteoporotic" effect of increased bone resorption due to increased diet acidity was shown to consist of osteocytic osteolysis and osteocytic chondrolysis. In the cortical bone of rats fed high acid diets (Figure 15A), the existing resorption cavities occurred in the center of individual osteons. These resorption cavities developed as

the result of bone resorption at the periphery of the osteons occurring at a faster rate than deposition at the osteonic centers, along with flow of bone toward the periphery. This process has been called osteocytic osteolysis (28). In the trabecular bone of rats fed acid-added diets (Figure 14C), the disappearance of chondroid core in the secondary spongiosa is an indication of osteocytic chondrolysis. In the primary spongiosa, the core is covered by bone and there are no free surfaces for osteoclasts or "chondroclasts" on which to act. The only cell available for removal of the core is the resorbing osteocyte. This process has been named osteocytic chondrolysis (305).

Despite the "osteoporotic" effect of diet acidity, the mineral composition of femurs of both age groups of rats in both trials (when these minerals were expressed as percent in ash) were unchanged by diet acid levels (Tables 30 and 36). This "osteoporosis" is a change in the amount of bone tissue rather than the chemical composition of bone tissue.

The overall conclusion from this study, therefore, is that high acid diets can cause osteopenia (a general bone loss) in rats, through increased osteocytic resorption of bone. However, chemically, the remaining bone (as a tissue) is unchanged by diet acidity.

B. Soft Tissue Mineralization

1. General

Changes in mineral concentrations in tissues in Mg-deficient animals have been documented in the literature

(101,102,271). Chemical alterations in tissues are characterized by increased Ca concentration and decreased Mg concentration in the heart, and increased Ca levels of the kidney. Bellavia et al. (35) showed that Mg also accumulates in renal tissue, probably due to codeposition with the Ca complexes. The present report confirmed these changes.

Woodard (313) observed nephrocalcinosis in young growing females, but not male, rats fed semipurified diets, which met all NRC requirements (227) and determined that the macromineral mixture was the dietary component causing nephrocalcinosis. Hurley et al. (142) showed that low Mg (0.040%) diets fed to female rats cause a trend of high kidney Ca. Martindale and Heaton (210) made a similar observation. The present study shows kidney calcification occurring in young-growing female rats fed 0.041-0.053% diet Mg (Tables 44, 48). Such findings are indicative of Mg deficiency, even though previous work has shown 0.040% diet Mg to be a level that provides optimum growth (179) and normal tissue Mg concentrations (214). The faster growing animals are those which may be expected to exhibit the more marked Mg deficiency symptoms (101). The present study shows that pathologic levels of kidney Ca can occur in Mg-deficient young growing rats in the absence of clinical signs such as hyperexcitability or skin hyperemia (Tables 44, 48).

2. Effects of Calcium

It is difficult to suggest the nature of the preventive action of Ca on renal calcinosis. There is abundant

evidence that one consequence of Mg deficiency in animals is a disturbance of Ca metabolism. Since parathyroid hormone (PTH) is a major factor controlling Ca metabolism, it is not surprising that hypotheses regarding alterations in parathyroid gland (PT) activity in Mg deficiency have been proposed (239). Both the cellular influx and efflux of Ca are facilitated by PTH (50). Borle (49) observed that purified PTH enhances uptake of Ca by monkey kidney cells. Associated with this increased Ca uptake was an increase in cellular Ca and Ca turnover. Rasmussen et al. (257) proposed that PTH activates adenyl cyclase, and that the resulting increase in cyclic AMP (cAMP) increases the permeability of the cell membrane to Ca++. Since the PTH-induced cellular efflux of Ca predominates over the influx (50), it would seem that depressing PT activity should increase renal Ca by decreasing efflux. However, Jowsey et al. (153) showed that secondary hyperparathyroidism causes soft tissue calcification. Furthermore, prolonged intravenous infusion of pure PTH in young rats produced severe nephrocalcinosis; this effect was blocked by simultaneous administration of calcitonin (CT) (256). Thus it would seem that depressing PT activity or counteracting the effects of PTH would actually decrease kidney calcification.

High diet Ca can depress PT activity (258) and CT can prevent renal calcification (94). Nevertheless, Mg deficiency in the rat seems associated with increased CT secretion (258), and it has been suggested that Mg deficiency is accompanied

by decreased PT activity, based on observations in Mg-deficient cattle, sheep, dogs, monkeys, man and chickens (239). The rat may be unique since many reports on this species have indicated that Mg deficiency is associated with increased PT activity, based on hypercalcemia, hypophosphatemia, and hyperphosphaturia (239). In addition, removal of the PT (in the rat) prevents any rise in kidney Ca concentration (130).

Hyperparathyroidism, however, has not been clearly established during Mg deficiency in rats based on cAMP excretion data. Cyclic AMP excretion may be presumed to reflect PT activity in the young rat. On this basis, Parker and Forbes (239) concluded that: (1) Mg deficiency reduces PT activity while P excess does not affect it; (2) both treatments induce kidney calcification; and (3) Ca deficiency increases PT activity irrespective of Mg status, although nephrocalcinosis appears only in Mg-deficient animals. Their data support the view that nephrocalcinosis of diet origin in the rat is not mediated by increased PT activity (239).

We are left, then, with a paradox. On the one hand, the PT must somehow be involved in kidney calcification, since the kidneys of parathyroidectomized Mg-deficient rats fail to calcify. The cAMP excretion data, on the other hand, suggest that the Mg-deficient rat has, if anything, less PTH output than normal animals (239). Furthermore, feeding extra Ca, to lower PT activity prior to Mg deficiency, does not prevent calcification (258). It therefore seems doubtful that

hyperactivity of the PT during Mg deficiency induces kidney calcification. In fact, recent histological evidence showed the PT in Mg-deficient rats to be hypoactive in response to hypercalcemia (152).

Another possible suggestion for the nature of preventive action of Ca on renal calcinosis is the modification of the Ca:P ratio in the urine. Experimentally induced ovine phosphatic urolithiasis can be reduced by raising the level of diet Ca, which lowers the urine P level (62). Magnesium deficiency has been shown to increase P urinary excretion (in normal or parathyroidectomized rats) and to decrease Ca urinary excretion (258). Addition of Ca decreases P urinary excretion and increased Ca excretion (258). It has therefore been suggested that modification of the Ca:P ratio in urine prevents kidney calcification during Mg deficiency (258). The fact that KK mice are genetically more susceptible to Mg deficiency-induced renal calcification (explicable by a lowered threshold level of the Ca/P product in the crystal formation of Ca phosphate salts) provides further support for this suggestion. Supplemental F inhibits the rise of the concentration product, and partly prevents the development of renal calcification. The action of F is based on a depressed urinary P excretion plus a dilution of the excreted Ca and P by a F-induced polyuria (141).

Still another mode of action in the inhibition of Mg deficiency-induced nephrocalcinosis by increased diet Ca may involve the replacement of Ca^{++} for Mg^{++} in non-pathologic

tissues as demonstrated in the present study (Tables 45 and 49). Increasing diet Ca would therefore increase normal tissue Ca^{++} concentrations, thereby lowering Mg^{++} levels in the tissue and consequently freeing Mg^{++} to reduce the influence of diet Mg deficiency.

3. Effects of Systemic Acidosis and Alkalosis

Glassman et al. (114) showed that NH_4Cl-induced metabolic acidosis in the dog decreases proximal tubular reabsorption of Ca. Similarly, Lemann et al. (193) showed that chronic metabolic acidosis produces increased urinary Ca excretion by decreasing Ca reabsorption. They suggested that this is a direct effect of metabolic acidosis on metabolic processes within renal tubular cells. Beck et al. (26) showed that in the kidney, acute metabolic acidosis directly inhibits the tubular reabsorption of Ca, but augments the increased tubular reabsorption of Ca caused by PTH. Transbol et al. (296) showed that alkalosis increases intestinal absorption of Ca and probably the tubular reabsorption of Ca.

Kaye (156) showed that chronic metabolic acidosis depresses PT activity, while alkalosis stimulates PT activity. It has also been shown that in acute metabolic acidosis serum Ca levels are elevated (26) while in chronic metabolic acidosis they are decreased (191).

These data may partly explain the observed different effects of diet acidity on kidney calcification in the two trials of the present study. In the Ca-depleted, Mg-deficient, young rats of Trial 1 which developed a more severe kidney

calcification on the acid-added diets (Table 47), serum Ca levels were also decreased as compared to those fed the non acid-added diets (Table 40). These would be the expected effects of chronic metabolic acidosis (191), due to depressed PT activity (156) and reduced renal tubular reabsorption of Ca (114,193).

In Trial 2, however, diet acidity had no effect on serum Ca levels (Table 43) although both acidosis and alkalosis resulted in less severe nephrocalcinosis in the young-growing rats (Table 50). The effects of alkalosis are the expected ones in as much as tubular reabsorption of Ca would be increased both as a direct effect of alkalosis on renal tubular cells (296), and as an indirect effect of the stimulation of the PT (156). Acidosis in this latter trial did not have the chronic effect of lowering serum Ca values, but may have augmented the effect of PTH to increase tubular reabsorption of Ca (26) and thus reduce the severity of nephrocalcinosis.

4. Mechanisms Involved in Nephrocalcinosis

It has been suggested that acute renal shutdown in nephrocalcinosis is the result of cellular injury secondary to intense renal tubular accumulation of Ca phosphate complexes (145). Furthermore, the state of renal ground substance has been implicated in the initiation of this Ca accumulation (16,259,279).

The ground substance is the extracellular amorphous matrix interspersed between the tubular cells and it is the medium through which metabolites and ions are continuously exchanged

(146). The specific identity of the renal ground substance is thought to be either glycoprotein or mucopolysaccharide (or both) which are capable of binding Ca and P in the renal tubules when altered (60).

The involvement of sulfated mucopolysaccharides in the process of calcification has been demonstrated in several investigations related to atherosclerosis and aortic plaque formation (82,103,195). The involvement of a sulfated mucopolysaccharide ground substance in the process of renal calcification due to prolonged Mg deficiency has also been reported (60,146). This involvement was indicated by sulfur incorporation in the kidneys. Initiation of Mg deficiency-induced renal calcification, however, was reported to precede any change in sulfate uptake by the kidney tissue (146). This would suggest that the initial increase in kidney Ca is not related to alteration in the renal ground substance and therefore not extracellularly initiated.

Electron microscopic studies have shown that the initial Ca deposits in kidneys of Mg-deficient rats are found either within lysosome-like bodies or free in the cytoplasm of kidney tubule cells (271). Another study showed that tubular nephrosis begins by changes in the basement membrane of kidney tubule cells (179). The present study also shows that kidney calcification is an intracellularly initiated process, beginning with the basement membrane (Figure 4C), later involving the tubular epithelial cells and eventually leading to the complete disintegration of the involved tubules

(Figure 4D). This confirms that low diet Mg-induced nephrocalcinosis is dystrophic calcification rather than just precipitation of Ca salts in the tubular lumen. It therefore appears reasonable to conclude that Ca accumulation commences intracellularly in the very early stages after a Mg deficiency begins, possibly due to the altered ionic composition of the tubular fluid. With the continued passage of this abnormal tubular fluid through the extracellular substance, it is possible that the physical components are changed, causing degenerative changes predisposing to further extensive calcification (156).

C. Cholesterolemia

Serum cholesterol was measured in both Trials 1 and 2. In Trial 1, serum cholesterol of the depleted rats of both age groups was lower than in the repleted rats (Table 37). There are several possible explanations for this effect. Tadayyon et al. (293) has reported that a low Ca:P ratio in the diet will result in a decreased absorption of the long chain saturated triglycerides. Several investigators (292, 293) have suggested that when this excess P (in relation to Ca) is present in the alimentary tract, a Ca phosphate-fatty acid complex is formed and excreted in the feces.

Another explanation involves the report by Cheng et al. (66) that diet Mg reduces the digestibility of high melting point fats. The Mg level (0.041-0.053%) of the depletion-repletion diets has been shown in this study to be pathologically low. It is therefore possible that the continued

feeding of this low level of diet Mg during the repletion period reduced the normal limiting effect of diet Mg on fat digestion, thereby resulting in a higher rate of fat absorption and an increased level of serum cholesterol in the repleted rats, compared to the depleted controls.

There is no certain explanation for the effect of diet Ca on serum cholesterol and cholesterol metabolism. However, various investigators seem to agree on the effect of diet Ca on fat absorption. Excess Ca in the diet depresses absorption of fat in rats (54,66,99,100,314), rabbits (144), chicks (95), lambs (76), dairy heifers (69), steers (7), infants (52) and adult humans (23,81,201,315). It is suggested that the ingested fat may be hydrolyzed to free fatty acids, forming insoluble Ca soaps which are excreted in the feces (117,293). It has also been reported that when either Ca or P intake is high in relation to the other, the absorption of the long chain saturated triglycerides is decreased (293).

The serum cholesterol data of the present study are in agreement with these findings. In Trial 1, when the diet Ca:P ratio was either low (0.22%:0.44%) or high (0.78%:0.44%), the serum cholesterol values of rats of both age groups (Table 38) were lower than when the diet Ca:P ratio was nearly equal (0.48%:0.44%). The same was true in Trial 2 where 0.78% diet Ca resulted in lower serum cholesterol values than 0.48% diet Ca in both age groups (Table 42). This same relationship was reported in another study with rats where 0.44% diet Ca resulted in higher serum

cholesterol values than either 0.18%, 0.69% or 0.98% diet Ca (266).

In Trial 1, increasing diet protein from 18 to 36% resulted in decreased serum cholesterol values in rats of both age groups (Table 39). These data are in agreement with other studies which have shown reduced serum cholesterol with increased diet protein levels in the chick (194), Cebus monkey (208) and rats (1,127). This effect may be a result of changes in the composition of the lipoprotein moiety of the serum, since it has been shown that at lower levels of protein, there is an increase in the percentage of lipid bound to the B-lipoproteins of serum (194). However, we are left with a paradox since 9% diet protein also resulted in decreased serum cholesterol values in rats of both age groups, compared to those fed 18% diet protein (Table 39). This may be a protein X Ca interaction. The serum cholesterol values for rats fed 9% diet protein are averaged across two diet Ca levels, 0.22% and 0.78%, which represent either a low (0.22%:0.44%) or high (0.78%:0.44% diet Ca:P ratio. As stated above, both a low or high diet Ca:P ratio results in decreased serum cholesterol levels. On the other hand, the serum cholesterol values for rats fed 18% diet protein represent a nearly equal (0.48%:0.44%) diet Ca:P ratio which has been shown to result in increased serum cholesterol, as stated above.

Another explanation to this paradox may involve the Ca-depleted status of the Trial 1 rats. The present study has shown that, in the Ca-depleted rat, increasing diet protein

from 9 to 18% stimulates the rate of bone turnover and growth. This would in turn increase the demand for Ca being absorbed through the gut, thereby reducing the amount of available Ca for forming insoluble Ca soaps in the gut. On the other hand, the excessive increase of diet protein from 18 to 36% has been shown in the present study to reduce bone resorption in the young rat and retard bone deposition and remodeling in the old rat. These latter effects would decrease the demand for Ca absorption through the gut, thereby increasing the amount of available Ca for forming insoluble Ca soaps.

In Trial 1, acid addition to the diet resulted in decreased serum cholesterol values in rats of both age groups (Table 40). A possible explanation of this effect may be related to the inverse relationship between serum Mg and cholesterol levels seen in this trial (Table 40). Such an inverse relationship between the levels of Mg and cholesterol has previously been reported in the serum of man (38) and rat (249). Furthermore, it has been shown that parenteral administration of Mg sulfate to patients with coronary thrombosis improves their abnormal lipoprotein pattern (205).

In Trial 2, increased diet acidity from pH 6.6 to pH 5.0 also resulted in decreased serum cholesterol values in rats of both age groups, but without an inverse effect on serum Mg (Table 43). No explanation is offered for this difference.

In Trial 2, increased diet alkalinity from pH 6.6 to

pH 7.4 resulted in decreased serum cholesterol values in rats of both age groups (Table 43). This may be due to an effect of the alkaline diet reducing the efficiency of absorption of Ca from the gut (5,90,207), thereby increasing the availability of Ca for forming insoluble Ca soaps.

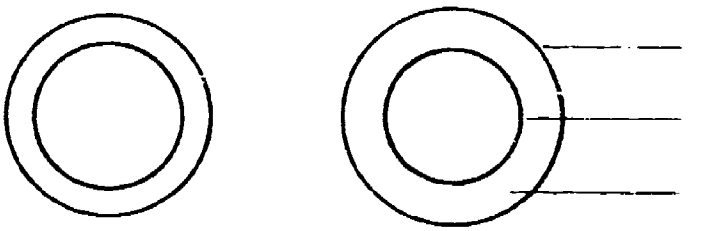
Old rats have been shown to have higher serum cholesterol values than young rats (266). The present study confirms this finding (Tables 37 and 41) and demonstrates that this effect is independent of diet regimen.

Figure 5.

A. Increased subperiosteal (T) and endosteal (M) deposition shown in Trial 1 repleted young rats compared to the Ca-depleted controls (Table 16). Cortical thickness (C), cortical area (CA) and percent cortical area (PCA) are increased by repletion.

B. Increased subperiosteal (T) deposition and endosteal (M) resorption shown in Trial 2 young test rats compared to the initial controls (Table 20). Cortical thickness (C), cortical area (CA) and percent cortical area (PCA) are absolutely increased. These changes all represent normal bone growth with aging.

A.


Depletion
Repletion
T increased
0.080 mm
M decreased
0.082 mm
C increased
0.162 mm
CA increased
0.66 mm2
PCA increased
5.7%

B.

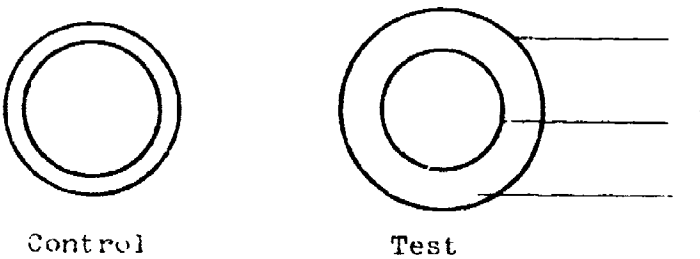

Control
Test
T increased
0.509 mm
M increased
0.273 mm
C increased
0.237 mm
CA increased
1.47 mm2
PCA increased
3.1%

Figure 6.

A. Femur transverse section of a Ca-depleted young rat (#A6). Large resorption cavities (horizontal arrows) and a thin subperiosteal lamellar bone layer (vertical arrow) are evident. H & E, X110.

B. Femur transverse section of a Ca-repleted young rat (#C35). Thickened lamellar bone at both surfaces (vertical arrows), abundant cementing lines (horizontal arrows) and retained chondroid core are evident. H & E, X110.

C. Femur longitudinal section of a Ca-depleted young rat (#A6). Thin trabeculae (arrow) are evident. H & E, X75.

D. Femur longitudinal section of a Ca-repleted young rat (#B14). Thick trabeculae (horizontal arrow) and considerable retained chondroid core (vertical arrow) are present in the diaphyseal secondary spongiosa. H & E, X75.

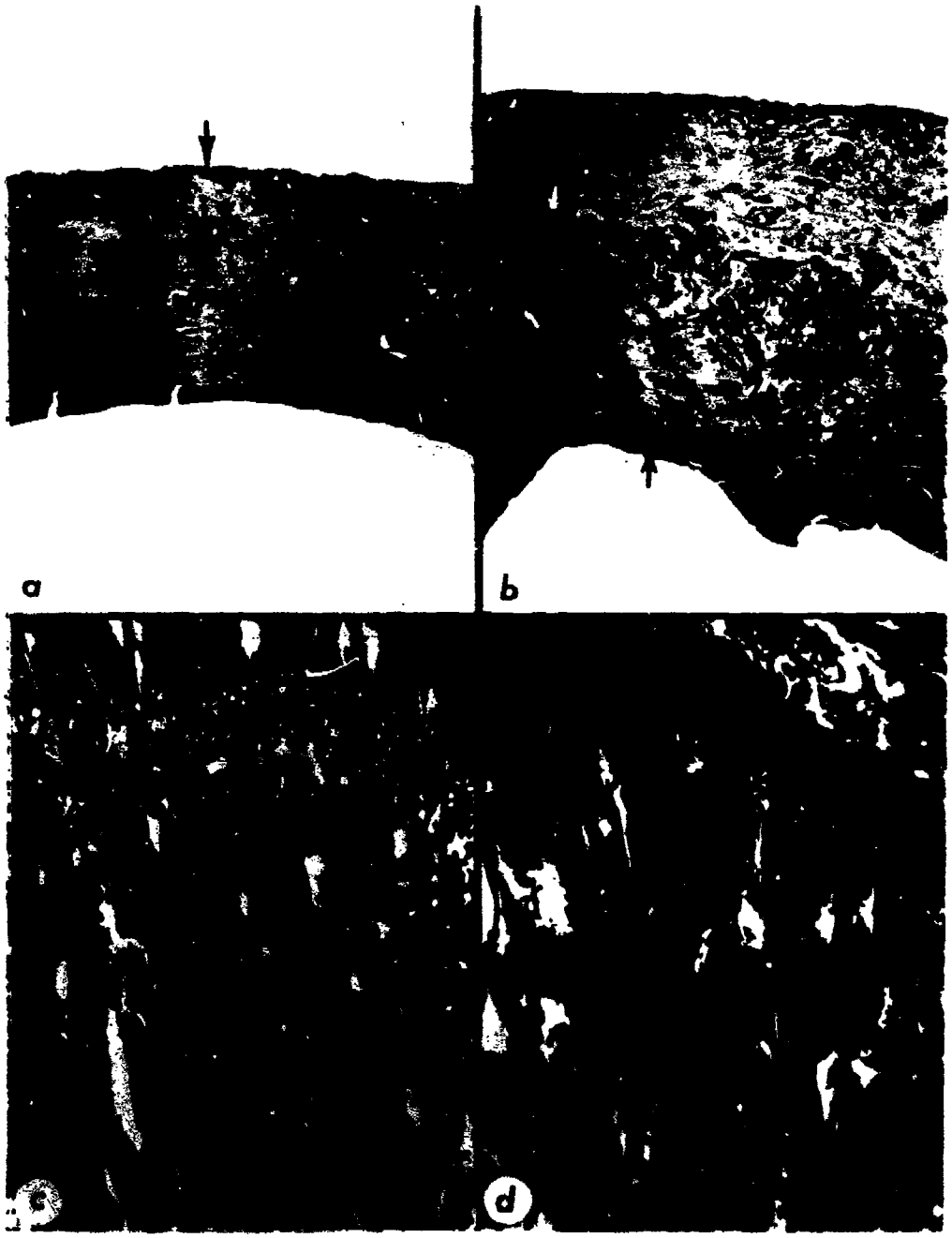

a
b
c
d

Figure 7.

A. Femur transverse section of a Trial 2 control young rat (#K23). The thick subperiosteal lamellar bone layer, numerous cementing lines (vertical arrow) and considerable retained chondroid core (horizontal arrow) are evident in the thin cortex. H & E, X110.

B. Femur transverse section of a Trial 2 experimental young rat (#L35). The thick subperiosteal lamellar bone layer, numerous cementing lines (vertical arrow) and considerable retained chondroid core (horizontal arrow) are evident in the thick cortex. H&E, X110.

C. Femur longitudinal section of a Trial 2 control young rat (#K23). Thin sparse trabeculae (vertical arrow) with considerable retained chondroid core (horizontal arrow) are evident. H & E, X75.

D. Femur longitudinal section of a Trial 2 experimental young rat (#L35). Thick trabeculae with considerable retained chondroid core (arrow) are evident. H & E, X75.

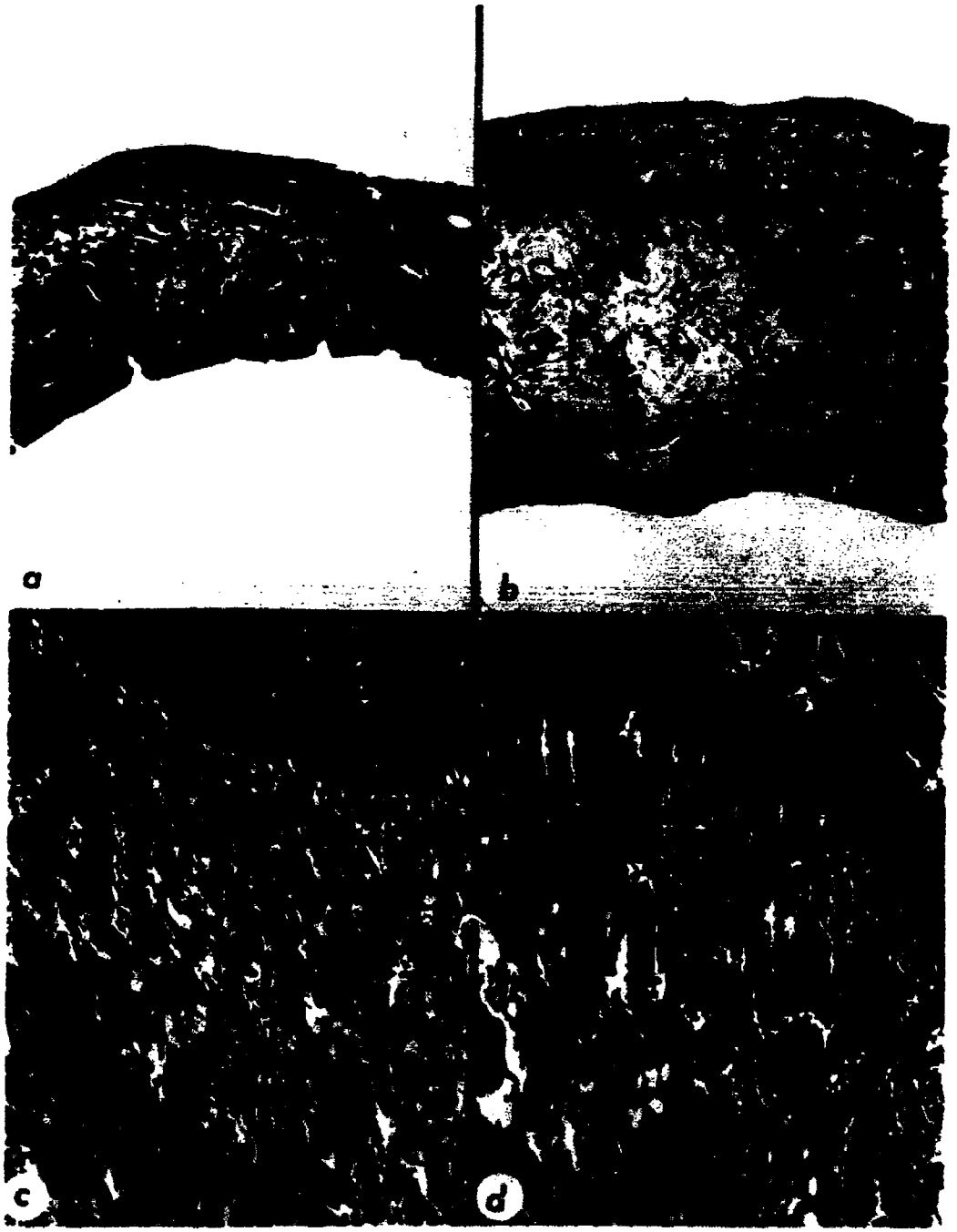
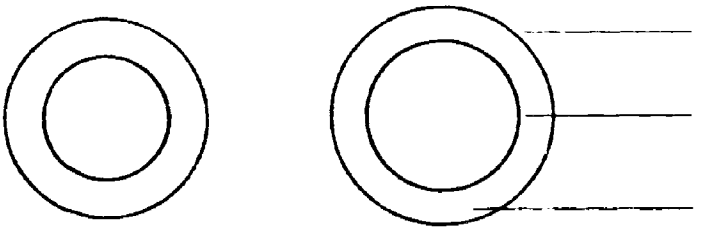

a
b
c
d

Figure 5.

A. Increased subperiosteal (T) deposition and increased endosteal (M) resorption shown in Trial 1 young rats fed a 0.78% Ca diet compared to young rats fed a 0.22% Ca diet (Table 17). Increased bone turnover with the increased diet Ca resulted in greater cortical area (CA), but a thinner cortex (C) and reduced percent cortical area (PCA).

B. Increased subperiosteal (T) deposition and increased endosteal (M) resorption shown in Trial 1 young rats fed a 36% protein diet compared to young rats fed a 9% protein diet (Table 18). Increased bone turnover with the increased diet protein resulted in greater cortical area (CA), but a thinner cortex (C) and reduced percent cortical area (PCA).

A.


0.22% Ca
0.78% Ca
T increased
0.175 mm
M increased
0.231 mm
C decreased
0.055 mm
CA increased
0.13 mm2
PCA decreased
4.7%

B.

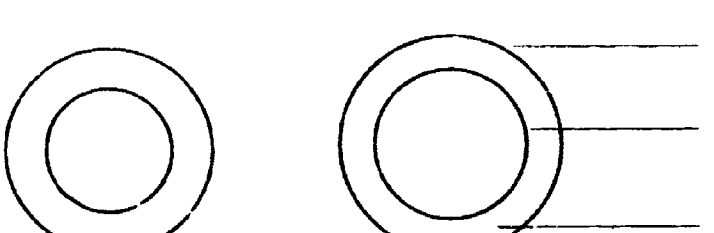

9% Protein
36% Protein
T increased
0.150 mm
M increased
0.173 mm
C decreased
0.077 mm
CA increased
0.19 mm2
PCA decreased
3.1%

Figure 9.

A. Femur transverse section of a Trial 1 young rat (#A8) fed a 0.22% Ca diet. Occasional cementing lines (vertical arrow) and retained chondroid core (horizontal arrow) are evident. H & E, X110.

B. Femur transverse section of a Trial 1 young rat (#B26) fed a 0.78% Ca diet. Numerous cementing lines (vertical arrow) and considerable retained chondroid core (horizontal arrow) are evident. H & E, X110.

C. Femur longitudinal section of a Trial 1 young rat (#A1) fed a 0.22% Ca diet. Thin sparse trabeculae (horizontal arrows) with very little retained chondroid core (vertical arrow) are evident. H & E, X75.

D. Femur longitudinal section of a Trial 1 young rat (#B14) fed a 0.78% Ca diet. Thick trabeculae (horizontal arrows) with considerable retained chondroid core (vertical arrow) are evident. H & E, X75.

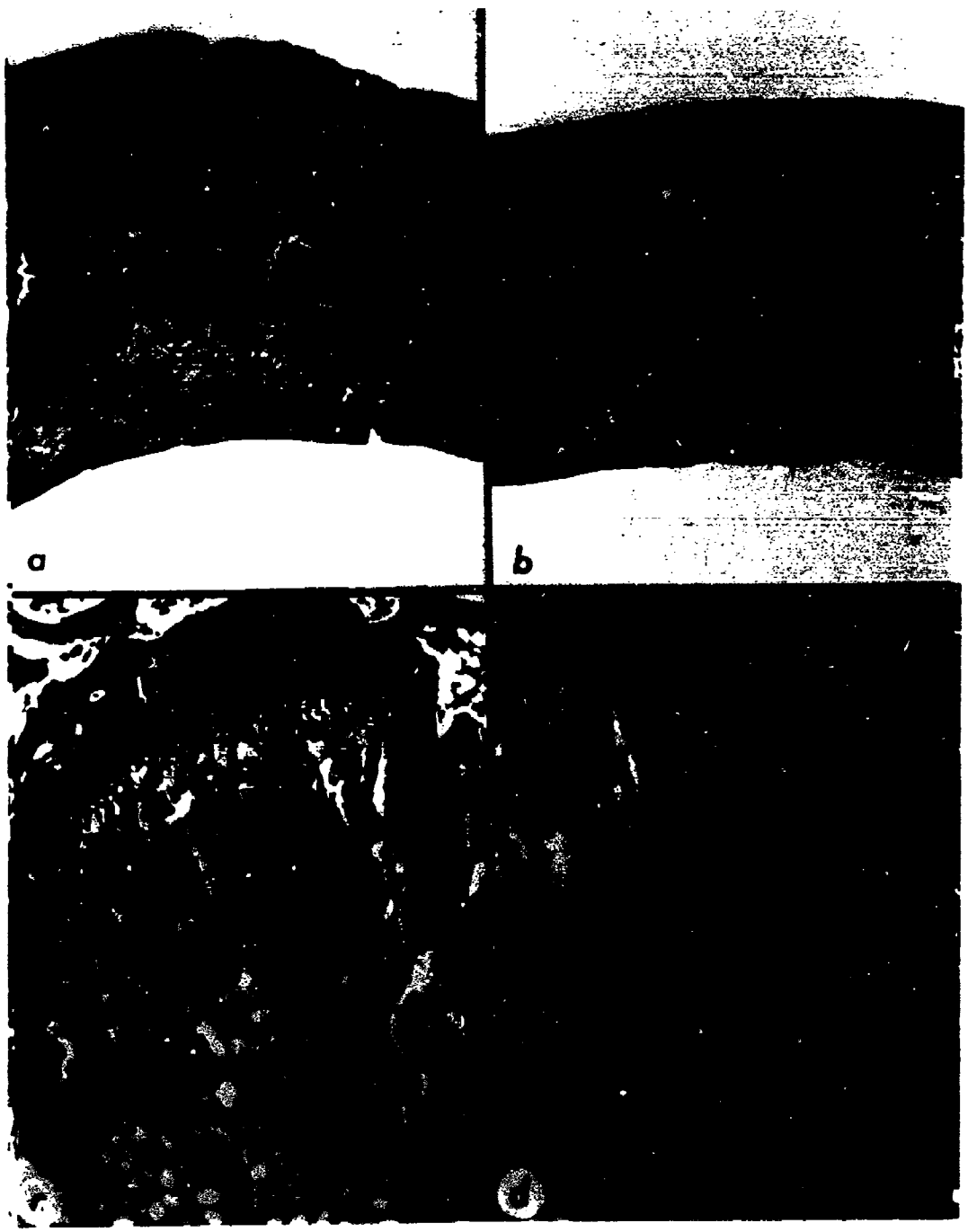

a
b

Figure 10.

A. Femur transverse section of a Trial 2 young rat (#L39) fed a 0.48% Ca diet. Numerous cementing lines (vertical arrow) and considerable retained chondroid core (horizontal arrow) are evident. H & E, X110.

B. Femur transverse section of a Trial 2 young rat (#L31) fed a 0.78% Ca diet. Numerous cementing lines (vertical arrow) and considerable retained chondroid core (horizontal arrow) are evident. H & E, X110.

C. Femur longitudinal section of a Trial 2 young rat (#L37) fed a 0.48% Ca diet. Thick trabeculae (horizontal arrow) with considerable retained chondroid core (vertical arrow) are evident. H & E, X75.

D. Femur longitudinal section of a Trial 2 young rat (#L38) fed a 0.78% Ca diet. Thick trabeculae (horizontal arrow) with considerable retained chondroid core (vertical arrow) are evident. H & E, X75.

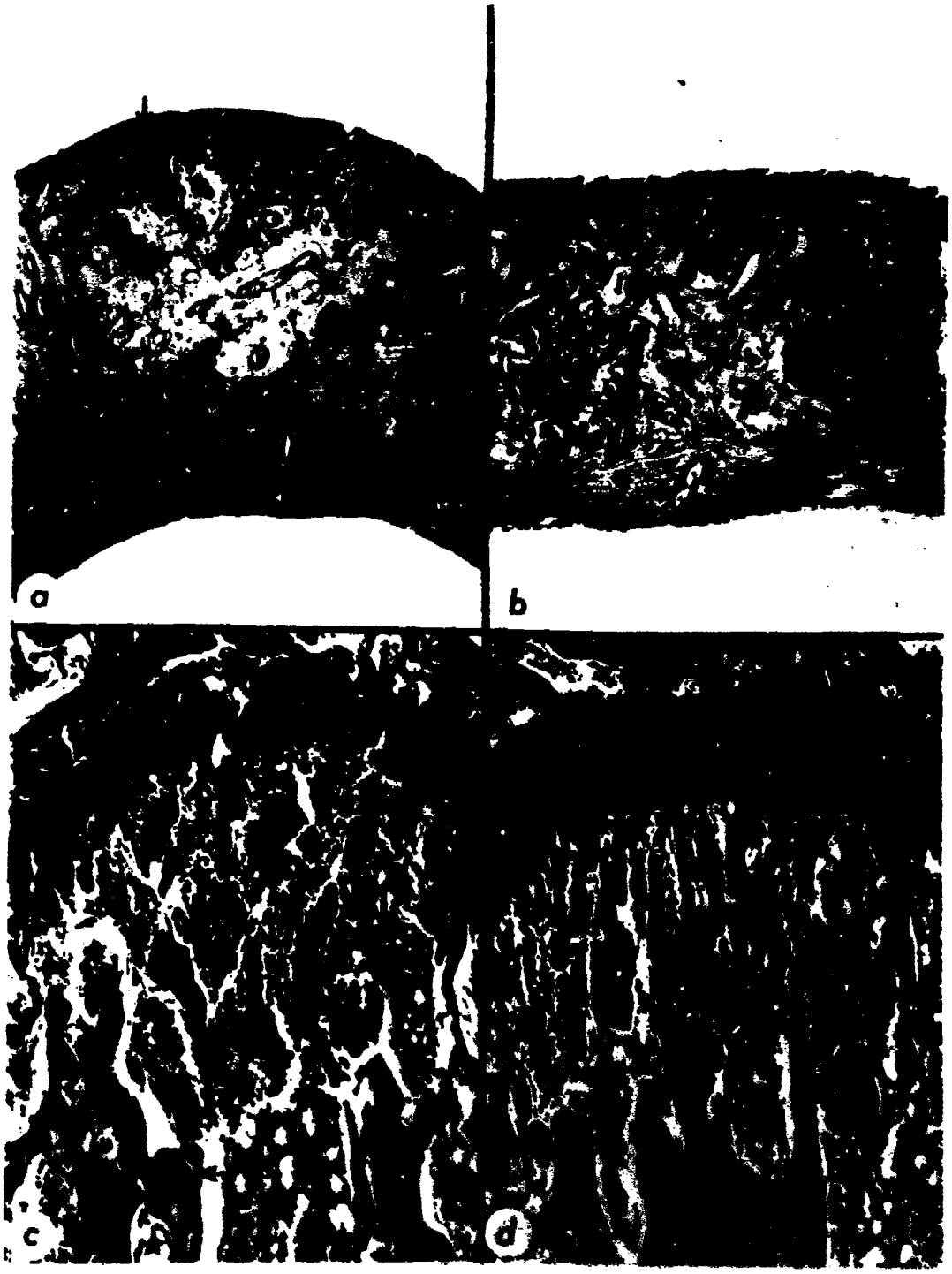

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Figure 11.

A. Femur transverse section of a Trial 1 young rat (#C37) fed a 9% protein diet. No cementing lines or retained chondroid core are present. H & E, X110.

B. Femur transverse section of a Trial 1 young rat (#C35) fed a 36% protein diet. The thick subperiosteal lamellar bone layer (between vertical arrows), numerous cementing lines (horizontal arrow) and considerable retained chondroid core are evident. H & E, X110.

C. Femur longitudinal section of a Trial 1 young rat (#C37) fed a 9% protein diet. Thin trabeculae (arrows) with no retained chondroid core are evident. H & E, X75.

D. Femur longitudinal section of a Trial 1 young rat (#C35) fed a 36% protein diet. Thick trabeculae (horizontal arrows) with considerable retained chondroid core (vertical arrow) are evident. H & E, X75.

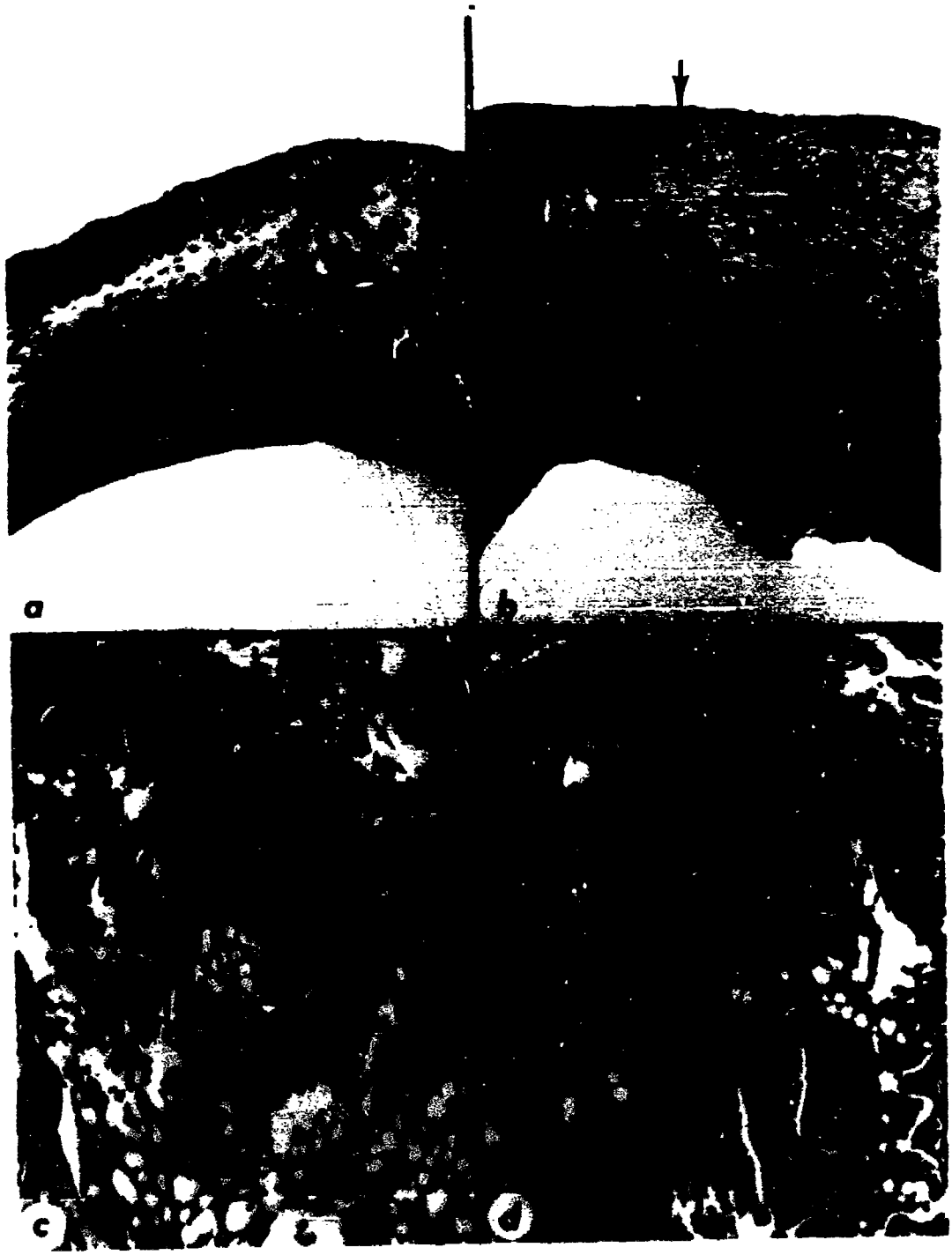
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Figure 12.

A. Femur transverse section of a Trial 1 old rat (#E58) fed a 9% protein diet. No cementing lines or retained chondroid core are present. H & E, X110.

B. Femur transverse section of a Trial 1 old rat (#H93) fed an 18% protein diet. Numerous cementing lines (vertical arrow) and retained chondroid core (horizontal arrow) are evident. H & E, X110.

C. Femur transverse section of a Trial 1 old rat (#G87) fed a 36% protein diet. The thick unremodeled endosteal lamellar bone layer (arrow) is evident. H & E, X110.

D. Femur longitudinal section of a Trial 1 old rat (#H95) fed a 9% protein diet. The thin trabeculae (arrows) with no retained chondroid core are evident. H & E, X75.

E. Femur longitudinal section of a Trial 1 old rat (#H99) fed an 18% protein diet. The thick trabeculae (horizontal arrow) with considerable retained chondroid core (vertical arrow) are evident. H & E, X75.

F. Femur longitudinal section of a Trial 1 old rat (#F66) fed a 36% protein diet. The thin, sparse and irregular trabeculae (arrow) are evident. H & E, X75.

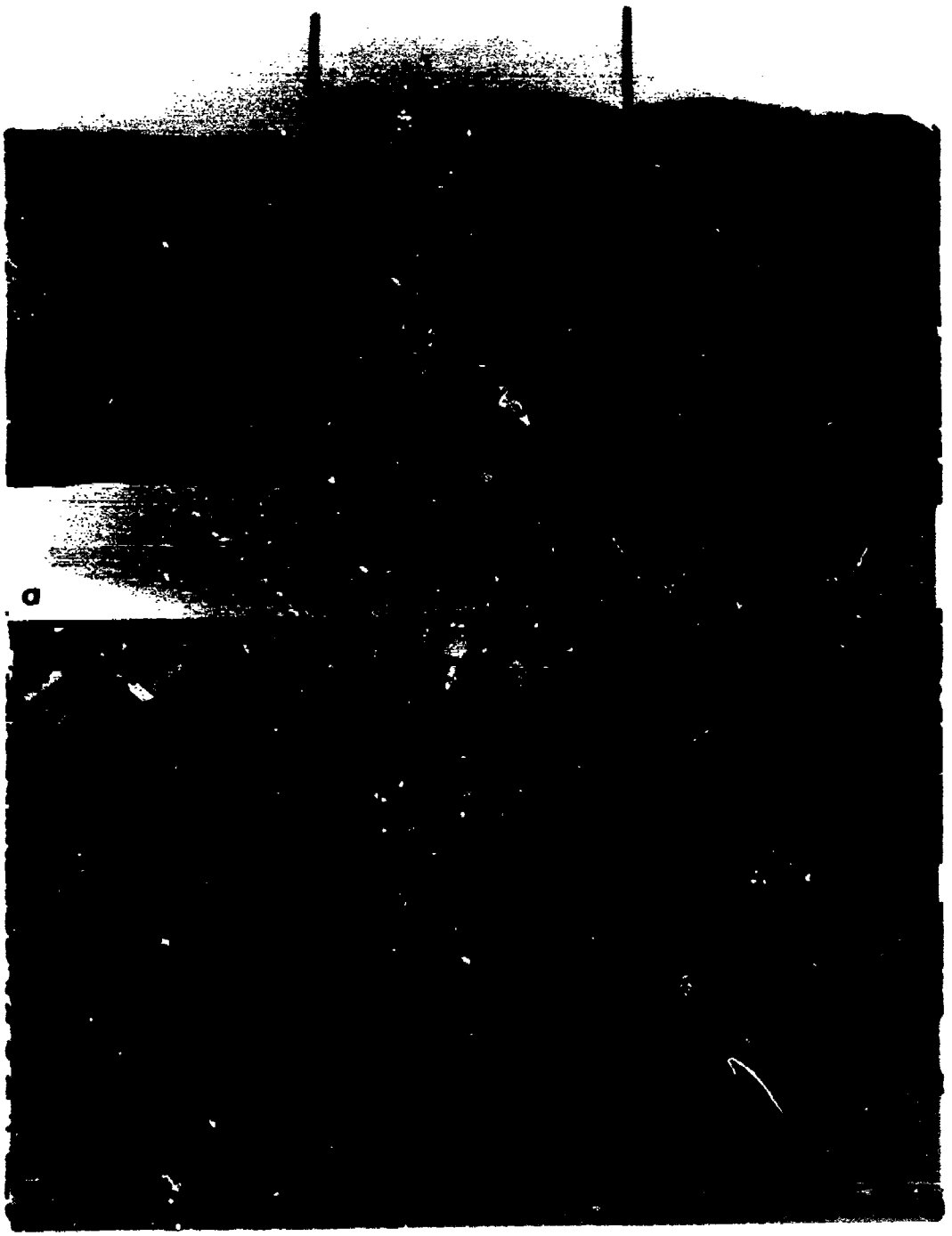
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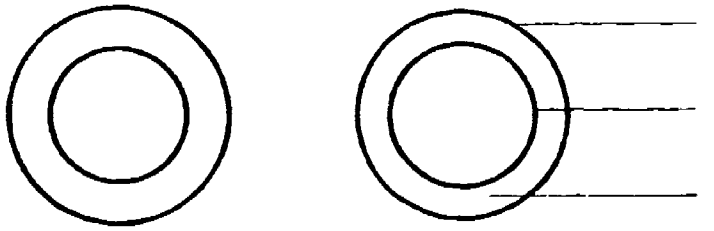
Figure 13.

A. Decreased subperiosteal (T) deposition and increased endosteal (M) resorption shown in Trial 1 young rats fed an acid-added diet compared to young rats fed a non acid-added diet (Table 19). Cortical thickness (C), cortical area (CA) and percent cortical area (PCA) were reduced as a result of increased diet acidity.

B. Decreased subperiosteal (T) deposition and increased endosteal (M) resorption shown in Trial 2 young rats fed a pH 5.0 diet compared to young rats fed a pH 7.4 diet (Table 22). Cortical thickness (C), cortical area (CA) and percent cortical area (PCA) were reduced as a result of increased diet acidity.

A.



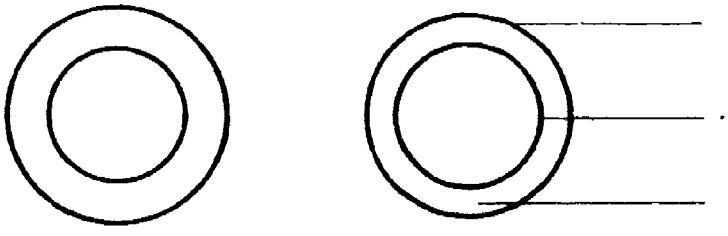
Non Acid-Added

Acid-Added

CA decreased
0.14 mm^2

PCA decreased
1.4%

B.



pH 7.4

pH 5.0

CA decreased
0.43 mm^1

PCA decreased
3.5%

Figure 14.

A. Femur transverse section of a Trial 1 young rat (#D41) fed an acid-added diet. There is a thin lamellar bone layer at both the subperiosteal and endosteal surfaces (arrows). Very few cementing lines and retained chondroid core are present. H & E, X110.

B. Femur transverse section of a Trial 1 young rat (#B25) fed a non acid-added diet. There is a thick lamellar bone layer at both the subperiosteal and endosteal surfaces (vertical arrows). Numerous cementing lines (horizontal arrow) and considerable retained chondroid core are evident. H & E, X110.

C. Femur longitudinal section of a Trial 1 young rat (#D41) fed an acid-added diet. The thin sparse trabeculae (arrows) with no retained chondroid core are evident. H & E, X75.

D. Femur longitudinal section of a Trial 1 young rat (#F75) fed a non acid-added diet. The thick trabeculae (horizontal arrows) with considerable retained chondroid core (vertical arrow) are evident. H & E, X75.


a
b
c
d

Figure 15.

A. Femur transverse section of a Trial 2 young rat (#L39) fed a pH 5.0 diet. Numerous resorption cavities (arrows) are present. H & E, X110.

B. Femur transverse section of a Trial 2 young rat (#L32) fed a pH 5.8 diet. Numerous resorption cavities (horizontal arrow) and an occasional cementing line (vertical arrow) are evident. H & E, X110.

C. Femur transverse section of a Trial 2 young rat (#L31) fed a pH 6.6 diet. Numerous cementing lines (horizontal arrows) and some retained chondroid core are evident. H & E, X110.

D. Femur transverse section of a Trial 2 young rat (#L34) fed a pH 7.4 diet. There is a greatly thickened lamellar bone layer at both the subperiosteal and endosteal surfaces (vertical arrows). Extensive cementing lines and an abundance of retained chondroid core (horizontal arrows) are evident. H & E, X110.

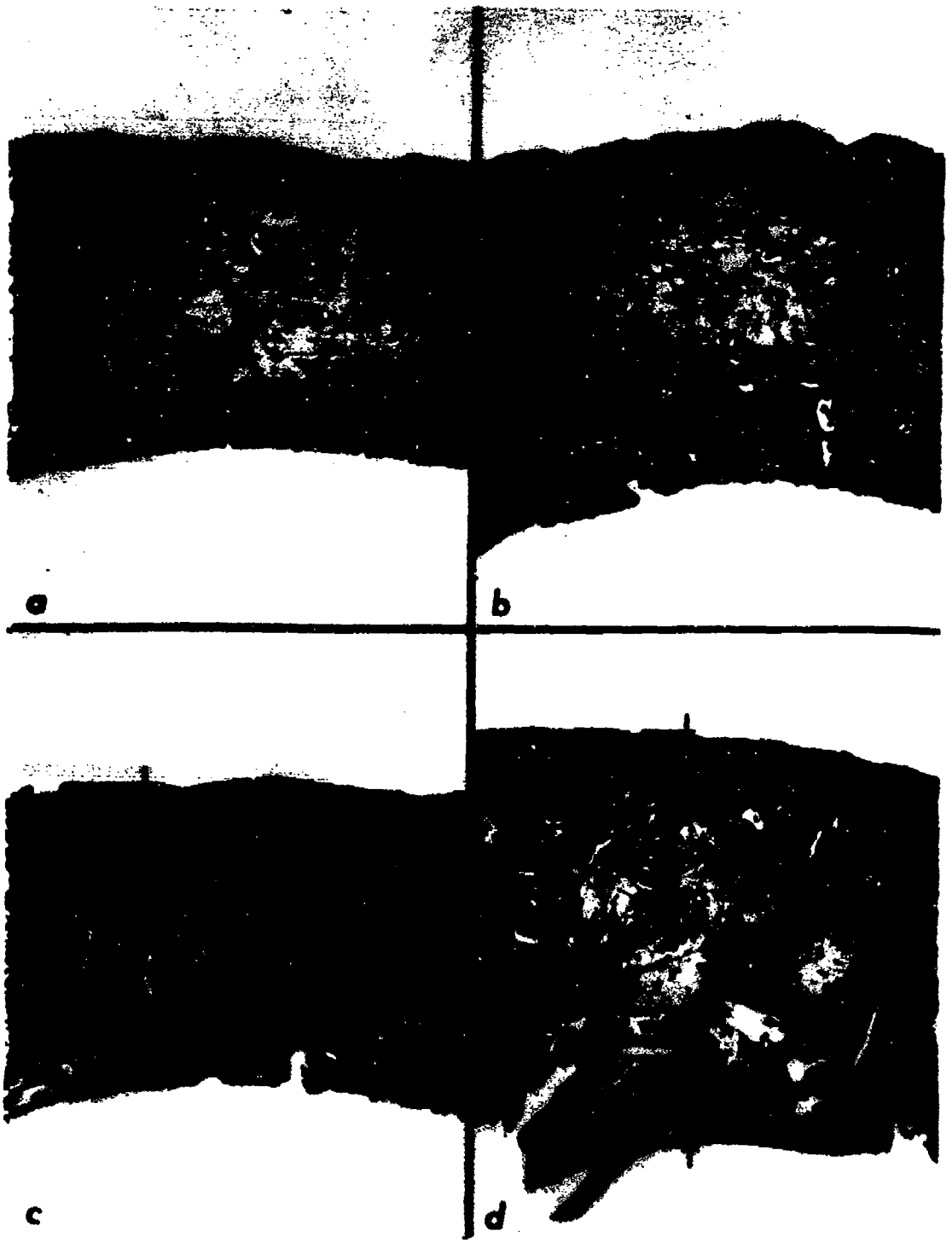

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Figure 16.

A. Femur longitudinal section of a Trial 2 young rat (#L39) fed a pH 5.0 diet. Very thin and sparse trabeculae (arrows) are evident. H & E, X75.

B. Femur longitudinal section of a Trial 2 young rat (#L32) fed a pH 5.8 diet. Moderately thick trabeculae (horizontal arrows) with a small amount of retained chondroid core (vertical arrow) are evident. H & E, X75.

C. Femur longitudinal section of a Trial 2 young rat (#L31) fed a pH 6.6 diet. The thick trabeculae (horizontal arrows) with considerable retained chondroid core (vertical arrows) are evident. H & E, X75.

D. Femur longitudinal section of a Trial 2 young rat (#L34) fed a pH 7.4 diet. The diaphysis is extensively laced with trabecular bone (horizontal arrows) which contains an abundant amount of retained chondroid core (vertical arrow). H & E, X75.

VI. SUMMARY

1. Calcium depletion produces osteopenia ("osteoporosis") in the young rat, through the mechanisms of increased bone resorption and decreased bone deposition.

2. Calcium repletion of the young rat overcomes this "osteoporotic" condition through increased bone deposition and decreased resorption.

3. Increasing the repletion diet Ca level from 0.22 to 0.78% improves the degree of recovery from "osteoporosis" in young rats.

4. Old rats are more refractory to changes in diet Ca, probably due to a smaller exchangeable Ca pool in the bone of older animals. Nevertheless, increasing the repletion diet Ca level from 0.22 to 0.78% increases bone density in old rats. This finding gives hope that, despite the refractoriness of mature bone, a diligent program of Ca supplementation might overcome the effects of "osteoporosis" in older individuals.

5. Serum HP may be a useful tool for the early diagnosis of "osteoporosis" in the aged when other clinical signs are still negative.

6. With increased diet Ca, femur K percent in ash is decreased in both young and old rats. This probably represents a cation exchange or a decrease in the extracellular fluid content and/or matrix of bone with increased mineralization.

7. With the exception of K, bone (as a tissue) is chemically unaffected by diet treatment. Age, on the other hand, creates some significant differences in femur mineral composition. These differences include higher femur Ca, Mg, Na, Cu, Fe and Zn and lower femur K and Mn percent in ash with increased age.

8. Increasing diet protein results in increased bone turnover at both bone surfaces, as well as increased CA and bone density in young rats. Total cortical thickness, PCA and CI, however, are reduced. Therefore, in the young growing rat, maximal skeletal growth rate stimulated by high diet protein may be incompatible with optimal skeletal characteristics.

9. Excess diet protein results in osteopoenia in old rats. This undesirable skeletal characteristic is not detectable by radiogrammetry or mineral analyses.

10. High acid diets cause "osteoporosis" (a general bone loss) in rats, through increased osteocytic resorption of bone. However, chemically, the remaining bone (as a tissue) is unchanged by diet acidity.

11. Magnesium deficiency characterized only by subclinical nephrocalcinosis can be induced in young growing female rats fed 0.041-0.053% diet Mg.

12. Increased diet Ca decreases the severity of Mg deficiency-induced nephrocalcinosis.

13. Severe chronic metabolic acidosis (as seen in Trial 1 of the present study), characterized by decreased serum Ca,

perpetuates the severity of Mg deficiency-induced nephrocalcinosis.

14. A less severe chronic metabolic acidosis (as seen in Trial 2 of the present study), characterized by normal serum Ca levels, reduces the severity of Mg deficiency-induced nephrocalcinosis.

15. Chronic metabolic alkalosis (as seen in Trial 2 of the present study) reduces the severity of Mg deficiency-induced nephrocalcinosis.

16. Magnesium deficiency-induced nephrocalcinosis is an intracellularly-initiated dystrophic calcification.

17. Clearly, more information about the mechanism of renal and other soft tissue calcification is needed. Once these mechanisms are fully understood, it might be possible to therapeutically or even nutritionally block these mechanisms and prevent nephrocalcinosis, rather than treat the clinical syndrome after it has occurred.

18. When the diet Ca:P ratio is either low or high, the serum cholesterol levels of both young and old rats are lower than when the diet Ca:P ratio is nearly equal.

19. Excess diet protein results in decreased serum cholesterol levels in both young and old rats.

20. Diet acidity has a quadratic effect on serum cholesterol levels in both young and old rats.

21. Old rats have higher serum cholesterol levels than young rats.

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VIII. VITA

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1941	Born August 16 in Grandin, New Jersey.
1959	Graduated from North Hunterdon Regional High School, Annandale, New Jersey.
1959-63	Attended College of Agriculture, Rutgers University, New Brunswick, New Jersey: Majored in Agriculture.
1963	B.S. in Agriculture, Rutgers University.
1963	Commissioned as "Reserve" Second Lieutenant, USAF.
1964-	Active duty, USAF.
1967	Promoted to Captain, USAF.
1970	Commissioned as "Regular" Captain, USAF.
1973-74	Attended Cook College, Rutgers University; Majored in Pre-Vet.
1974-75	Graduate work in Animal Science, Rutgers University.
1975	M.S. in Animal Science, Rutgers University.
1975	Promoted to Major, USAF.
1975-79	Attended New York State College of Veterinary Medicine, Cornell University, Ithaca, New York.
1975-79	Graduate work in Nutrition, Rutgers University.
1978	Abstract: "Osteopenia and Soft Tissue Calcification as Affected by Diet Calcium and Acidity," Fed. Proc. 37:847.
1979	Abstract: "Effects of Diet Calcium and Acidity on Low Diet Magnesium - Induced Nephrocalcinosis in Rats," Fed. Proc. 38:765.
1979	Article: "A Survey of Waste Anesthetic Gases in Selected USAF Veterinary Surgeries," USAF OEHL TR-79-33, USAF OEHL, AFSC/AMD, Brooks AFB, Texas.
1979	D.V.M., Cornell University.
1979	Ph.D. in Nutrition, Rutgers University.

IX. APPENDIX

The tables in the Appendix tabulate all the data collected in this study. An explanation of the headings used in these tables is as follows:

OBS	Observation number.
AGE	Age of rat, mature (M) or young (Y).
CAL	Diet calcium, %.
PROT	Diet protein, %.
A	Diet acidity, acid-added (A) or natural (N).
PH	Diet pH.
RG	Rat group replication number.
RNO	Individual rat number.
AD	Femur air dry weight, mg.
H_2O	Femur weight in water, mg.
VOLCC	Femur volume, cc.
SPGR	Femur specific gravity, unit.
FFD	Femur fat-free dry weight, mg.
FFD/AD	FFD/AD, %.
ASH	Femur ash, mg.
ASH/AD	ASH/AD, %.
ASH/FFD	ASH/FFD, %.
ASH/cc	Femur ash/cc, mg.
MGCA	Femur calcium, mg.
UGMG	Femur magnesium, ug.
T	Femur total subperiosteal diameter, mm.
M	Femur medullary cavity diameter, mm.
C	Femur total cortical thickness, mm.

CA	Femur cortical area, mm^2.
PCA	Femur percent cortical area, %.
CI	Femur cortical index, unit.
FCASHCA	Femur calcium percent in ash, %.
PCASHMG	Femur magnesium percent in ash, %.
PCASHNA	Femur sodium percent in ash, %.
PCASHK	Femur potassium percent in ash, %.
PCASHCU	Femur copper percent in ash, %.
PCASHFE	Femur iron percent in ash, %.
PCASHMN	Femur manganese percent in ash, %.
PCASHZN	Femur zinc percent in ash, %.
KIDCA	Kidney calcium, ppm.
KIDM	Kidney magnesium, ppm.
HCA	Heart calcium, ppm.
HMG	Heart magnesium, ppm.
SCA	Serum calcium, mg/100 ml.
SMG	Serum magnesium, mg/100 ml.
SHP	Serum hydroxyproline, ug/100 ml.
SC	Serum cholesterol, mg/100 ml.
SP	Serum protein, g/100 ml.
IW	Initial weight, g.
DW	Depletion weight, g.
RW	Repletion weight, g.
SW	Standardization weight, g.

Trial 1. Control

OBS	AGE	CAL	PROT	A	PH	RG	RNO	AD	H_2O	VOLCC	SPGR	FED	FED/AD
1	Y	0.16	18	N	5.8	0	3	613	161	0.4526	1.3557	351	57.3
2	Y	0.16	18	N	5.8	0	4	625	172	0.4537	1.3791	362	58.0
3	Y	0.16	18	N	5.8	0	6	668	187	0.4804	1.3907	391	58.6
4	Y	0.16	18	N	5.8	0	15	665	190	0.4750	1.4013	386	58.1
5	Y	0.16	18	N	5.8	0	21	683	195	0.4875	1.4014	398	58.3
6	Y	0.16	18	N	5.8	0	23	709	186	0.5232	1.3563	393	55.4
7	Y	0.16	18	N	5.8	0	27	779	277	0.5517	1.4129	469	60.2
8	Y	0.16	18	N	5.8	0	28	658	194	0.4640	1.4196	394	59.9
9	Y	0.16	18	N	5.8	0	34	651	192	0.4588	1.4194	388	59.7
10	Y	0.16	18	N	5.8	0	44	734	217	0.5169	1.4202	433	59.0
11	Y	0.16	18	N	5.8	0	48	682	193	0.4889	1.3960	401	58.8
12	Y	0.16	18	N	5.8	0	49	700	198	0.5019	1.3957	411	58.8
13	M	0.16	18	N	5.8	0	57	956	303	0.5632	1.5382	592	67.3
14	M	0.16	18	N	5.8	0	61	785	270	0.5150	1.5243	521	66.4
15	M	0.16	18	N	5.8	0	69	882	295	0.5871	1.5028	566	64.2
16	M	0.16	18	N	5.8	0	71	800	277	0.5236	1.5294	538	67.3
17	M	0.16	18	N	5.8	0	86	995	384	0.6114	1.5142	641	64.4
18	M	0.16	18	N	5.8	0	97	996	333	0.6627	1.5034	629	63.2
19	M	0.16	18	N	5.8	0	100	999	339	0.6595	1.5150	685	68.6
20	M	0.16	18	N	5.8	0	101	911	319	0.5925	1.5386	613	67.3
21	M	0.16	18	N	5.8	0	102	869	284	0.5848	1.4863	576	66.3
22	M	0.16	18	N	5.8	0	103	879	288	0.5915	1.4869	563	64.1
23	M	0.16	18	N	5.8	0	104	857	295	0.5616	1.5265	579	67.6
24	M	0.16	18	N	5.8	0	105	1034	351	0.6833	1.5141	674	65.2

Trial 1. Control (continued)

OBS	ASH	ASH/AD	ASH/FFD	ASH/CC	MGCA	PCASHCA	UGMG	PCASHMG	T	M	C
1	200	32.7	57.0	442	67	33.8	996	0.50	3.115	2.135	0.980
2	206	33.0	57.0	455	71	34.6	1074	0.52	3.040	2.155	0.885
3	232	34.9	59.4	484	78	33.6	1258	0.54	3.015	1.980	1.035
4	226	34.0	58.5	476	80	35.6	1143	0.51	3.090	1.905	1.185
5	234	34.3	58.8	480	76	32.7	1152	0.49	3.200	2.260	0.940
6	234	33.0	59.6	448	75	32.3	1154	0.49	3.145	2.185	0.960
7	283	36.4	60.4	513	92	32.5	1408	0.50	3.275	2.325	0.950
8	237	36.1	60.3	512	76	32.2	1160	0.49	3.120	2.240	0.880
9	233	35.9	60.1	508	73	31.6	1157	0.50	3.080	2.010	1.070
10	262	35.8	60.7	508	82	31.3	1273	0.48	3.080	2.120	0.960
11	231	33.9	57.7	473	72	31.4	1103	0.48	3.095	2.120	0.975
12	237	33.9	57.6	472	72	30.4	1238	0.52	3.115	2.125	0.990
13	381	44.0	65.5	677	125	33.0	2659	0.70	3.435	2.230	1.205
14	343	43.7	65.8	666	111	32.4	2531	0.74	3.390	2.040	1.350
15	373	42.3	65.9	635	119	31.9	2590	0.69	3.380	2.115	1.265
16	354	44.3	65.8	677	111	31.5	2407	0.68	3.125	1.730	1.395
17	402	40.4	62.7	657	117	29.3	2632	0.65	3.595	2.275	1.320
18	409	41.1	65.1	618	120	29.3	2641	0.64	3.370	1.940	1.430
19	464	46.4	67.7	703	133	28.9	2635	0.57	3.605	2.170	1.435
20	410	45.0	66.9	692	118	28.9	2635	0.64	3.450	2.155	1.295
21	386	42.4	64.0	630	122	31.7	2358	0.61	3.375	2.090	1.285
22	352	40.0	62.5	595	104	29.5	2408	0.68	3.560	2.345	1.215
23	383	44.7	66.1	682	113	29.5	2641	0.69	3.525	2.275	1.250
24	448	43.4	66.5	657	135	30.1	2700	0.60	3.640	2.245	1.395

Trial 1. Control (continued)

OBS	CA	PCA	CI	KIDCA	KIDM	HCA	HMG	SCA	SMG	SHP	SC	SI	IW	DW
1	4.04	53.0	0.315	22909	1213	356	854	10.4	2.1	490	38	3.9	79	242
2	3.61	49.7	0.291	10782	813	351	933	10.2	2.1	706	75	4.2	90	257
3	4.06	56.9	0.343	18178	1026	247	812	9.3	1.8	600	38	4.2	82	239
4	4.65	62.0	0.383	12281	983	294	784	10.9	2.6	475	42	4.5	95	233
5	4.03	50.1	0.294	28706	1243	292	855	10.1	2.3	653	19	4.6	98	233
6	4.02	51.7	0.305	27869	1092	378	871	10.1	2.0	925	16	3.8	98	224
7	4.16	49.6	0.290	14901	961	243	788	11.2	2.2	700	96	4.6	100	252
8	3.70	48.5	0.282	26139	1221	249	827	10.4	2.8	547	80	4.2	101	236
9	4.28	57.4	0.347	8669	813	256	892	10.4	2.6	750	96	4.4	104	242
10	3.92	52.6	0.312	23924	1190	293	881	10.4	2.2	650	47	4.3	107	235
11	3.99	53.1	0.315	10956	826	310	865	9.9	2.4	736	95	5.4	108	256
12	4.07	53.5	0.318	6467	798	289	920	10.6	2.3	828	94	4.2	109	246
13	5.36	57.8	0.351	210	657	257	832	10.0	2.4	339	156	4.6	264	289
14	5.76	63.7	0.398	266	693	378	859	10.1	2.1	288	94	4.4	268	280
15	5.46	60.3	0.374	208	629	236	845	10.3	2.3	332	68	4.6	277	297
16	5.32	69.3	0.446	189	665	251	872	10.6	2.2	331	125	4.9	279	307
17	6.08	59.9	0.367	211	695	228	837	10.3	2.1	415	90	5.0	297	347
18	5.96	66.8	0.424	225	630	276	814	10.1	2.2	342	60	4.2	313	330
19	6.51	63.7	0.398	211	568	247	811	10.3	2.0	480	186	5.0	333	382
20	5.70	60.9	0.375	263	650	245	864	11.3	2.7	470	147	4.6	288	337
21	5.51	61.6	0.381	265	708	234	803	10.3	2.5	223	127	4.8	272	309
22	5.63	56.6	0.341	236	615	292	795	10.9	2.9	364	96	4.7	285	313
23	5.69	58.3	0.355	230	646	301	811	10.9	2.8	388	97	4.6	305	334
24	6.45	61.9	0.383	227	692	268	879	10.0	2.6	247	90	5.8	330	341

Trial 1. Experimental

OBS	AGE	CAL	PROT	A	PH	RG	RNG	AD	H_2O	VOLCC	SPGR	FFD	FFD/AD
1	Y	0.78	9	A	5.2	A	1	791	243	0.5478	1.4451	503	63.6
2	Y	0.22	9	A	5.2	A	2	705	212	0.4923	1.4321	425	60.3
3	Y	0.78	36	A	5.2	A	5	819	254	0.5651	1.4502	514	62.7
4	Y	0.22	36	N	5.4	A	7	782	245	0.5368	1.4581	493	63.0
5	Y	0.22	36	A	5.2	A	8	818	247	0.5711	1.4325	503	61.4
6	Y	0.48	18	A	5.2	A	9	776	243	0.5336	1.4554	474	61.0
7	Y	0.48	18	N	5.8	A	10	802	248	0.5538	1.4482	509	63.5
8	Y	0.78	36	N	5.6	A	11	789	254	0.5358	1.4742	518	65.6
9	Y	0.78	9	N	6.0	A	12	769	241	0.5278	1.4581	500	64.9
10	Y	0.22	9	N	6.0	A	13	777	243	0.5339	1.4559	505	65.0
11	Y	0.78	9	N	6.0	B	14	834	264	0.5700	1.4644	537	64.3
12	Y	0.22	9	A	5.2	B	16	735	225	0.5099	1.4419	452	61.6
13	Y	0.22	36	A	5.2	B	17	731	226	0.5053	1.4473	452	61.9
14	Y	0.48	18	A	5.2	B	18	803	245	0.5575	1.4409	498	62.1
15	Y	0.22	9	N	6.0	B	19	690	210	0.4796	1.4397	448	64.9
16	Y	0.78	9	A	5.2	B	20	719	226	0.4925	1.4605	465	64.7
17	Y	0.78	36	N	5.6	B	22	803	254	0.5485	1.4640	514	64.0
18	Y	0.22	36	N	5.4	B	24	787	237	0.5499	1.4321	502	63.7
19	Y	0.48	18	N	5.8	B	25	764	242	0.5218	1.4653	495	64.7
20	Y	0.78	36	A	5.2	B	26	767	247	0.5200	1.4760	509	66.4
21	Y	0.78	9	A	5.2	C	29	772	233	0.5382	1.4344	469	64.2
22	Y	0.48	18	N	5.8	C	30	864	305	0.5885	1.4683	542	62.8
23	Y	0.22	9	N	6.0	C	31	815	247	0.5677	1.4365	491	60.2
24	Y	0.22	36	A	5.2	C	32	749	222	0.5263	1.4231	455	60.8
25	Y	0.48	18	A	5.2	C	33	789	251	0.5381	1.4665	497	63.1
26	Y	0.78	36	N	5.6	C	35	829	271	0.5577	1.4866	532	64.2
27	Y	0.22	36	N	5.4	C	36	770	237	0.5334	1.4447	488	63.3
28	Y	0.22	9	A	5.2	C	37	717	203	0.5140	1.3961	434	60.5
29	Y	0.78	36	A	5.2	C	38	814	260	0.5535	1.4712	526	64.6
30	Y	0.78	9	N	6.0	C	39	841	281	0.5594	1.5038	533	63.4
31	Y	0.22	36	N	5.4	D	40	772	243	0.5296	1.4592	494	64.0
32	Y	0.48	18	A	5.2	D	41	811	251	0.5593	1.4502	512	63.1

Trial 1. Experimental (continued)

OBS	AGE	SAL	PROT	A	PH	PG	RND	AD	H_2O	VOLCC	SPGR	FFD	FFD/AD
33	Y	0.48	18	N	5.8	D	42	773	241	0.5369	1.4492	490	63.0
34	Y	0.22	9	N	6.0	D	43	710	216	0.4944	1.4378	442	62.9
35	Y	0.78	36	A	5.2	D	45	777	247	0.5308	1.4664	498	64.1
36	Y	0.78	36	N	5.6	D	46	833	266	0.5669	1.4705	536	64.4
37	Y	0.78	9	N	6.0	D	47	767	235	0.5322	1.4421	483	62.9
38	Y	0.22	36	A	5.2	D	50	766	237	0.5292	1.4489	486	63.4
39	Y	0.22	9	A	5.2	D	51	719	216	0.5028	1.4302	452	62.9
40	Y	0.78	9	A	5.2	D	52	765	237	0.5281	1.4492	495	65.1
41	M	0.48	18	N	5.8	E	53	781	266	0.5150	1.5167	518	65.8
42	M	0.78	9	A	5.2	E	54	963	327	0.6356	1.5154	629	65.3
43	M	0.78	36	N	5.6	E	55	880	296	0.5834	1.5086	569	64.7
44	M	0.78	36	A	5.2	E	56	947	312	0.6347	1.4920	610	64.4
45	M	0.22	9	A	5.2	E	58	782	253	0.5291	1.4782	512	65.5
46	M	0.22	9	N	6.0	E	59	729	245	0.4833	1.5088	497	68.1
47	M	0.78	9	N	6.0	E	60	852	307	0.5452	1.5642	574	67.3
48	M	0.22	36	A	5.2	E	62	923	315	0.6082	1.5186	614	66.4
49	M	0.22	36	N	5.4	E	63	815	260	0.5553	1.4682	532	64.0
50	M	0.48	18	A	5.2	E	64	907	283	0.6279	1.4459	592	65.2
51	M	0.22	36	N	5.4	F	65	914	300	0.6133	1.4906	597	65.3
52	M	0.22	36	A	5.2	F	66	927	301	0.6257	1.4823	597	64.3
53	M	0.78	9	N	6.0	F	67	863	302	0.5611	1.5389	589	68.1
54	M	0.22	9	A	5.2	F	68	826	275	0.5515	1.4988	541	65.5
55	M	0.78	36	N	5.6	F	70	926	302	0.6234	1.4859	598	64.6
56	M	0.48	18	N	5.8	F	72	931	326	0.6045	1.5401	622	66.8
57	M	0.78	36	A	5.2	F	73	905	282	0.6227	1.4537	602	66.6
58	M	0.48	18	A	5.2	F	74	928	318	0.6103	1.5212	607	65.3
59	M	0.22	9	N	6.0	F	75	846	288	0.5581	1.5160	620	66.5
60	M	0.78	9	A	5.2	F	76	913	303	0.6103	1.4966	600	65.7
61	M	0.48	18	N	5.8	G	78	965	329	0.6364	1.5174	643	66.6
62	M	0.48	18	A	5.2	G	79	894	290	0.6045	1.4797	572	64.0
63	M	0.22	36	N	5.4	G	80	902	300	0.6021	1.4991	582	64.5

Trial 1. Experimental (continued)

OBS	AGE	CAL	PROT	A	PH	RG	RNO	AD	H_2O	VOLCC	SPGR	PFD	PFD/AD
64	M	0.78	9	N	6.0	G	81	962	325	0.6372	1.5104	610	63.3
65	M	0.22	9	A	5.2	G	82	937	307	0.6302	1.4873	616	65.7
66	M	0.22	36	A	5.2	G	83	887	273	0.6138	1.4459	574	64.7
67	M	0.22	9	N	6.0	G	84	941	320	0.6210	1.5153	608	64.6
68	M	0.78	36	N	5.6	G	85	1027	344	0.6829	1.5043	663	64.6
69	M	0.78	36	A	5.2	G	87	915	307	0.6076	1.5066	604	65.9
70	M	0.78	9	A	5.2	G	88	945	302	0.6424	1.4714	620	65.6
71	M	0.22	36	N	5.4	H	89	878	281	0.5970	1.4712	564	64.2
72	M	0.22	9	N	6.0	H	90	947	322	0.6251	1.5154	619	65.4
73	M	0.22	9	A	5.2	H	91	1121	347	0.7733	1.4496	708	63.1
74	M	0.78	36	N	5.6	H	92	886	316	0.5702	1.5549	600	67.7
75	M	0.48	18	A	5.2	H	93	849	290	0.5596	1.5182	561	66.1
76	M	0.22	36	A	5.2	H	94	907	291	0.6162	1.4731	590	65.1
77	M	0.78	9	A	5.2	H	95	931	311	0.6196	1.5031	601	64.6
78	M	0.78	9	N	6.0	H	96	1014	348	0.6664	1.5224	675	66.5
79	M	0.78	36	A	5.2	H	98	970	334	0.6365	1.5249	646	66.6
80	M	0.48	18	N	5.8	H	99	954	329	0.6252	1.5267	641	67.2

Trial 1. Experimental (continued)

OBS	ASH	ASH/AD	ASH/FFD	ASH/CC	MGCA	PCASHCA	UCMG	PCASHMG	T	M	C
1	303	38.3	60.2	554	97	32.0	1377	0.45	3.170	2.045	1.125
2	253	35.9	59.5	514	86	33.9	1290	0.51	2.960	1.785	1.175
3	319	38.9	62.0	564	108	33.8	1497	0.47	3.425	2.315	1.110
4	301	38.4	61.0	561	96	31.9	1302	0.43	3.095	1.940	1.155
5	298	36.5	59.4	523	97	32.7	1377	0.46	3.185	2.000	1.185
6	302	38.9	63.7	566	104	34.6	1319	0.44	3.210	2.075	1.135
7	308	38.5	60.6	557	99	32.0	1440	0.47	3.190	2.010	1.180
8	323	40.9	62.3	603	103	31.8	1490	0.46	3.335	2.210	1.125
9	304	39.5	60.8	576	102	33.8	1394	0.46	3.125	1.925	1.200
10	308	39.6	60.9	577	103	33.7	1302	0.42	3.085	1.930	1.155
11	325	39.0	60.6	571	108	33.3	1359	0.42	3.265	2.125	1.140
12	271	36.9	60.0	532	91	33.7	1275	0.47	2.915	1.805	1.110
13	277	37.9	61.2	549	91	32.9	1299	0.47	3.185	2.040	1.145
14	301	37.4	60.3	540	99	33.0	1337	0.44	3.240	2.070	1.170
15	258	37.4	57.7	539	85	32.9	1293	0.50	3.115	1.870	1.245
16	283	39.3	60.8	575	95	33.7	1304	0.46	3.145	2.040	1.105
17	315	39.2	61.2	574	112	35.6	1446	0.46	3.440	2.305	1.135
18	301	38.3	60.1	548	103	34.1	1215	0.40	3.245	2.125	1.120
19	297	38.9	60.1	570	96	32.2	1287	0.43	3.215	2.040	1.175
20	315	41.1	61.9	607	98	31.0	1358	0.43	3.325	2.235	1.090
21	295	38.2	59.4	548	99	33.6	1373	0.47	3.205	2.175	1.030
22	328	38.0	60.5	558	118	35.9	1562	0.48	3.220	2.030	1.190
23	299	36.7	60.9	527	96	32.3	1396	0.47	2.965	1.735	1.230
24	269	35.9	59.1	512	83	30.9	1205	0.45	3.155	2.050	1.105
25	315	39.9	63.3	586	97	31.0	1610	0.51	3.240	2.105	1.135
26	331	40.0	62.2	594	108	32.8	1441	0.43	3.280	2.190	1.090
27	297	38.6	60.9	558	95	31.8	1427	0.48	3.235	2.020	1.215
28	256	35.7	59.0	498	79	31.1	1265	0.49	3.090	1.930	1.160
29	328	40.3	62.3	593	112	34.3	1576	0.48	3.305	2.240	1.065
30	333	39.6	62.5	596	110	33.1	1521	0.46	3.125	1.985	1.140
31	297	38.5	60.1	561	95	31.9	1473	0.48	3.170	1.960	1.210
32	314	38.7	61.3	561	93	29.8	1354	0.43	3.150	2.055	1.095

Trial 1. Experimental (continued)

OBS	ASH	ASH/AD	ASH/FFD	ASH/CC	MGCA	PCASHCA	UGMG	PCASHMG	T	M	C
33	308	39.6	62.9	574	104	33.7	1551	0.50	3.230	2.115	1.115
34	264	37.1	59.9	534	86	32.6	1192	0.45	3.015	1.925	1.190
35	308	39.7	61.9	582	102	33.0	1516	0.49	3.365	2.280	1.085
36	337	40.4	62.8	594	108	32.1	1529	0.45	3.385	2.260	1.125
37	296	38.6	61.3	557	99	33.3	1416	0.48	3.265	2.095	1.170
38	297	38.8	61.2	562	101	34.0	1300	0.44	3.095	1.930	1.165
39	263	36.6	58.2	524	88	33.5	1182	0.45	3.115	1.925	1.190
40	302	39.5	60.6	572	93	31.0	1381	0.46	3.265	2.130	1.135
41	342	43.8	66.6	665	108	31.7	2194	0.64	3.230	1.980	1.250
42	425	44.1	67.5	668	133	31.4	2805	0.66	3.055	1.795	1.260
43	369	42.0	64.9	634	121	32.7	2417	0.65	3.485	2.255	1.230
44	392	41.4	64.3	618	129	32.9	2544	0.65	3.395	2.170	1.225
45	325	41.6	63.5	615	101	31.1	2083	0.64	3.295	2.070	1.225
46	317	43.5	63.9	657	104	32.8	2009	0.63	3.430	2.137	1.295
47	376	44.1	65.5	690	121	32.1	2372	0.63	2.975	1.780	1.195
48	393	42.6	64.1	647	125	31.9	2587	0.66	3.430	2.160	1.270
49	333	40.9	63.9	601	101	30.3	1992	0.60	3.220	2.015	1.205
50	380	41.9	64.2	605	121	32.0	2473	0.65	3.330	2.065	1.265
51	384	42.0	64.3	626	125	32.7	2423	0.65	3.435	1.970	1.465
52	384	41.4	64.3	613	118	30.9	2506	0.65	3.355	2.045	1.310
53	391	45.2	66.4	696	127	32.5	2477	0.63	3.175	1.890	1.285
54	348	42.2	64.4	632	106	30.4	2233	0.64	3.095	1.900	1.195
55	390	42.1	65.1	625	118	30.4	2457	0.63	3.140	1.845	1.295
56	413	44.4	66.5	684	129	31.1	2812	0.68	3.625	2.380	1.245
57	386	42.7	64.1	621	111	28.8	2514	0.65	3.245	1.885	1.095
58	386	41.6	63.6	633	124	32.1	2668	0.69	3.260	2.055	1.205
59	363	43.0	64.6	652	107	29.6	2100	0.58	3.285	2.010	1.275
60	377	41.3	62.8	618	107	28.5	2529	0.67	3.185	1.840	1.345
61	419	43.4	65.1	658	136	32.6	2807	0.67	3.425	2.065	1.360
62	377	42.2	65.9	625	116	30.7	2510	0.66	3.535	2.265	1.270
63	372	41.3	64.0	619	118	31.7	2450	0.66	3.200	1.925	1.275

Trial 1. Experimental (continued)

OBS	ASH	ASH/AD	ASH/FFB	ASH/CC	MICA	PCASHCA	UGWG	PCASHGG	T	M	C
64	395	41.1	64.8	621	119	30.1	2493	0.63	3.540	2.300	1.240
65	393	41.9	63.8	624	126	32.2	2601	0.66	3.335	2.005	1.195
66	355	40.1	61.9	579	115	32.3	2248	0.63	3.313	2.010	1.110
67	385	40.9	63.3	620	121	31.6	2382	0.62	3.385	2.150	1.235
68	440	42.8	66.3	644	139	31.7	2728	0.62	3.735	2.420	1.315
69	398	43.5	65.9	655	120	30.1	2682	0.67	3.535	2.360	1.175
70	385	40.7	62.1	599	118	30.8	2453	0.64	3.590	2.475	1.115
71	359	40.9	63.6	601	117	32.9	2442	0.68	3.440	2.260	1.180
72	402	42.4	64.9	643	126	31.5	2482	0.62	3.625	2.245	1.380
73	440	39.2	62.1	569	136	31.0	2730	0.62	3.720	2.385	1.285
74	400	45.1	66.6	702	123	30.9	2624	0.66	3.435	2.055	1.380
75	366	43.1	65.3	655	118	32.3	2384	0.65	3.375	2.000	1.375
76	375	41.4	63.6	609	115	30.6	2405	0.64	3.155	1.825	1.330
77	408	43.8	67.8	659	131	32.2	2531	0.62	3.400	2.035	1.365
78	448	44.2	66.4	673	142	31.6	2961	0.64	3.590	2.150	1.440
79	423	43.6	65.5	665	140	33.0	2783	0.66	3.360	1.990	1.135
80	413	43.3	64.4	661	130	31.5	2769	0.67	3.445	2.105	1.340

Trial 1. Exrerimental (continued)

OBS	CA	PCA	CI	KIDCA	KIDM	HCA	HM1	SCA	SMG	SHP	SC	SP	IW	DW	RW
1	4.61	58.3	0.355	9606	696	466	818	10.5	2.9	363	35	4.3	74	237	263
2	4.38	63.6	0.397	17240	911	392	851	9.5	3.3	389	109	4.4	75	240	278
3	5.00	54.3	0.324	5467	692	458	781	9.9	2.7	310	113	4.4	82	219	255
4	4.57	60.7	0.373	19914	815	307	859	9.6	2.2	319	111	5.4	85	254	295
5	4.82	60.5	0.372	16100	927	329	854	10.5	3.8	345	99	4.4	87	252	293
6	4.71	58.2	0.354	17345	810	400	757	9.9	2.8	345	73	4.9	90	210	262
7	4.82	60.3	0.370	8200	738	377	861	9.3	2.4	363	129	4.4	91	271	315
8	4.90	55.0	0.337	4038	735	438	832	10.5	2.4	276	141	4.9	91	198	251
9	4.76	62.0	0.384	3485	666	403	945	9.8	2.3	319	107	4.8	91	260	279
10	4.55	60.8	0.374	13405	835	278	912	11.0	2.8	337	111	5.2	92	251	295
11	4.82	57.6	0.349	3295	604	425	842	10.3	2.6	337	123	5.4	94	256	283
12	4.11	61.6	0.381	12473	875	305	905	9.6	2.7	423	126	5.1	95	244	277
13	4.70	58.9	0.359	16637	859	334	806	8.9	2.6	292	111	4.5	95	226	265
14	4.88	59.1	0.361	12788	871	385	808	9.9	2.5	337	121	4.4	96	245	276
15	4.87	63.9	0.400	12619	814	337	894	9.7	2.5	328	127	5.4	96	228	266
16	4.50	57.9	0.351	4337	676	481	795	10.6	3.0	345	135	4.2	98	225	250
17	5.12	55.1	0.330	4152	610	457	834	9.2	2.6	258	99	4.4	98	232	277
18	4.72	57.1	0.345	15146	846	374	771	11.2	3.0	276	134	4.6	99	247	289
19	4.85	59.7	0.365	13152	777	411	849	10.7	2.6	250	123	4.5	100	238	270
20	4.76	54.8	0.328	9413	682	406	838	9.1	2.9	241	123	4.1	100	228	255
21	4.35	53.9	0.321	8682	665	399	803	8.9	2.8	345	110	4.5	102	250	278
22	4.90	60.2	0.370	10463	794	364	872	10.6	2.5	354	145	5.2	102	236	256
23	4.54	65.7	0.415	16450	841	365	803	10.5	2.5	345	112	4.2	102	251	283
24	4.51	57.7	0.350	15269	863	364	815	9.7	3.3	292	138	4.5	103	248	278
25	4.76	57.7	0.350	11854	832	423	835	11.0	2.9	396	157	4.1	103	255	289
26	4.65	55.4	0.332	5769	691	438	798	10.1	2.8	267	130	4.8	104	258	287
27	5.01	61.0	0.376	20813	882	371	874	10.6	2.7	319	86	4.7	104	229	271
28	4.57	60.9	0.375	18499	843	325	847	8.4	2.5	397	93	3.9	105	214	248
29	4.64	54.0	0.322	8870	684	473	780	9.8	2.4	284	100	4.2	105	246	278
30	4.57	59.6	0.365	3536	651	462	809	10.4	2.5	328	99	4.2	105	226	258
31	4.87	61.7	0.382	14684	916	287	840	9.2	2.1	267	109	3.8	105	241	272
32	4.47	57.4	0.348	12204	808	372	860	10.1	2.5	354	124	4.4	105	268	284

Trial 1. Experimental (continued)

OBS	CA	FCA	CI	KIDCA	KIDM	HCA	HMG	SJA	JMG	SHP	SC	SP	IW	DW	RW
33	4.68	57.1	0.345	8872	747	360	809	11.4	2.5	302	113	4.4	105	252	281
34	4.52	63.3	0.395	15947	822	287	840	10.0	2.2	302	72	4.2	106	227	259
35	4.81	54.0	0.322	6598	772	419	831	9.6	2.5	267	63	4.8	107	239	269
36	4.99	55.4	0.332	4059	694	387	799	11.1	2.8	232	51	4.8	107	256	284
37	4.92	58.8	0.358	4448	676	378	862	11.0	2.7	284	71	4.4	105	254	267
38	4.60	61.1	0.376	21820	885	333	845	9.6	2.6	310	85	4.1	109	225	252
39	4.71	61.8	0.382	18426	895	327	817	10.4	2.7	397	56	4.3	111	248	289
40	4.81	57.4	0.348	9734	737	395	806	9.2	2.5	389	86	4.2	115	248	271
41	5.11	62.4	0.387	335	653	336	818	11.9	2.4	232	134	5.7	244	276	273
42	4.80	65.4	0.412	368	581	309	781	10.3	2.8	250	122	5.1	259	317	306
43	5.54	58.1	0.353	384	533	381	792	10.5	2.4	224	123	5.2	260	314	302
44	5.35	59.1	0.361	421	547	386	811	10.1	2.5	250	91	4.8	261	300	297
45	5.16	60.5	0.372	319	617	313	839	9.7	2.9	258	91	4.6	264	300	304
46	5.65	61.1	0.378	254	708	250	902	9.3	2.7	232	133	4.2	265	293	290
47	4.46	64.2	0.402	299	659	300	847	9.9	2.5	215	153	5.3	266	314	313
48	5.57	60.3	0.370	287	633	320	884	10.1	3.4	258	96	4.6	269	308	297
49	4.95	60.8	0.374	317	617	283	870	11.2	2.6	241	195	4.9	269	303	300
50	5.36	61.5	0.380	289	531	312	846	10.3	3.0	232	171	4.9	270	319	323
51	6.22	67.1	0.426	292	594	288	811	10.1	2.9	224	150	4.9	271	328	322
52	5.55	62.8	0.390	306	610	297	772	9.7	2.9	232	184	5.2	273	325	329
53	5.11	64.5	0.405	359	491	337	764	9.3	2.4	206	144	5.2	274	288	290
54	4.69	62.3	0.386	293	629	342	760	10.9	2.8	319	163	4.8	276	316	335
55	5.07	65.4	0.412	311	500	353	775	11.7	2.6	189	138	4.5	279	322	338
56	5.87	56.8	0.343	333	562	299	773	9.8	2.4	197	172	4.6	280	312	326
57	5.48	66.2	0.419	320	524	312	736	10.9	2.9	215	138	5.1	281	333	323
58	5.03	60.2	0.370	348	591	383	820	10.4	2.9	267	161	4.8	282	319	322
59	5.30	62.5	0.388	304	595	298	788	9.8	2.3	224	139	5.1	283	289	302
60	5.31	66.6	0.422	319	559	372	798	8.2	2.5	267	80	4.5	284	312	321
61	5.86	63.6	0.397	270	563	370	838	11.6	2.5	180	154	5.1	286	338	339
62	5.78	58.9	0.359	391	554	327	771	10.1	2.4	293	135	4.7	287	324	317
63	5.13	63.8	0.398	296	578	283	780	9.3	2.4	232	134	5.1	298	326	315

Trial 1. Experimental (continued)

OBS	CA	PCA	CI	KIDCA	KIDM	HCA	HMG	SCA	SMG	SHF	SP	TW	DW	RW
64	5.63	57.7	0.350	321	619	370	758	9.9	2.6	224	123	4.5	292	308	304
65	5.58	63.8	0.399	335	530	321	806	8.9	2.5	337	105	4.7	293	339	339
66	5.44	63.1	0.393	291	492	323	791	9.2	2.4	250	125	4.6	294	323	329
67	5.37	59.6	0.365	277	751	281	848	10.6	2.6	284	103	5.5	294	347	329
68	5.35	58.0	0.352	374	539	315	821	10.1	2.1	163	145	4.9	295	308	311
69	5.44	55.4	0.332	366	523	386	809	8.6	2.4	215	105	4.4	298	320	309
70	5.31	52.4	0.311	331	518	367	786	8.9	2.5	267	61	4.8	298	314	310
71	5.28	56.8	0.343	322	595	324	834	9.8	2.4	232	82	4.6	299	348	354
72	5.60	61.6	0.381	221	584	308	847	10.2	2.6	276	145	4.7	299	362	359
73	5.40	58.9	0.359	284	602	276	897	10.2	2.6	310	97	4.9	300	337	346
74	5.95	64.2	0.402	329	630	328	843	9.8	2.4	197	101	4.9	301	349	339
75	5.80	64.8	0.407	344	574	350	771	10.4	2.4	250	141	4.9	305	349	324
76	5.20	66.5	0.422	303	632	298	810	9.8	2.5	258	127	4.6	309	369	360
77	5.82	64.1	0.401	364	508	391	816	9.8	2.6	258	116	5.0	311	344	341
78	6.49	64.1	0.401	312	599	316	887	10.4	2.5	215	77	4.7	313	356	335
79	5.75	64.9	0.408	317	547	337	810	9.4	3.1	199	90	4.9	315	344	330
80	5.84	62.6	0.389	346	612	280	849	10.1	2.4	250	189	4.7	328	376	355

Trial 2. Control

OBS	AGE	CAL	PH	RG	RNO	AD	H_2O	VOLCC	SPGR	FFD	FFD/AD	ASH	ASH/AD	ASH/CC
1	M	0.48	5.8	C	3	891	261	0.6300	1.4146	629	70.7	407	45.7	646
2	M	0.48	5.8	C	10	707	174	0.5330	1.3280	480	67.8	302	42.8	566
3	M	0.48	5.8	C	13	807	218	0.5891	1.3706	548	67.9	351	43.5	596
4	M	0.48	5.8	C	23	820	222	0.5984	1.3710	554	67.6	353	43.1	591
5	M	0.48	5.8	C	31	652	195	0.4574	1.4263	460	70.6	300	46.1	657
6	M	0.48	5.8	C	36	965	282	0.6829	1.4138	671	69.5	429	44.5	628
7	Y	0.48	5.8	C	3	241	17	0.2238	1.0768	102	42.7	53	22.4	240
8	Y	0.48	5.8	C	10	383	68	0.3145	1.2178	148	38.8	71	18.7	228
9	Y	0.48	5.8	C	13	397	58	0.3390	1.1720	152	38.3	72	18.3	215
10	Y	0.48	5.8	C	23	364	95	0.2689	1.3544	151	41.6	75	20.6	279
11	Y	0.48	5.8	C	24	384	92	0.2916	1.3169	149	39.0	72	18.9	247
12	Y	0.48	5.8	C	30	416	114	0.3018	1.3797	169	40.6	85	20.5	282
13	Y	0.48	5.8	C	36	440	122	0.3180	1.3842	177	40.4	86	19.7	273

Trial 2. Control (continued)

OBS	T	M	C	CA	PCA	CI	PCASHCA	PCASHP	PCASHMG	PCASHNA	PCASHK	PCASHCL
1	3.520	2.110	1.410	6.23	64.1	0.401	40.1	22.6	0.79	1.64	1.23	0.0044
2	3.325	2.145	1.180	5.06	58.4	0.355	39.9	22.6	0.69	1.55	1.75	0.0073
3	3.380	2.090	1.290	5.53	61.9	0.382	38.8	22.4	0.68	1.40	1.32	0.0040
4	3.335	2.085	1.250	5.31	60.9	0.375	39.0	21.7	0.67	1.30	1.06	0.0045
5	3.640	2.320	1.320	6.17	59.4	0.363	38.5	21.1	0.59	1.16	0.94	0.0040
6	3.800	2.390	1.410	6.85	60.4	0.371	38.5	21.4	0.69	1.13	0.81	0.0023
7	2.325	1.550	0.775	2.35	55.6	0.333	42.1	23.9	0.55	1.17	1.62	0.0167
8	2.650	1.760	0.890	3.00	55.9	0.336	37.5	21.4	0.55	1.20	2.25	0.0042
9	2.675	1.695	0.980	3.36	59.8	0.366	38.5	22.3	0.50	1.24	3.28	0.0069
10	2.590	1.690	0.900	3.02	57.4	0.347	37.9	23.5	0.59	1.11	3.22	0.0040
11	2.475	1.725	0.750	2.47	51.4	0.303	37.5	23.2	0.55	1.46	4.22	0.0097
12	2.680	1.910	0.770	2.77	49.2	0.287	38.9	23.1	0.63	1.27	2.87	0.0035
13	2.770	1.960	0.810	3.00	49.9	0.292	39.2	23.8	0.59	1.72	4.34	0.0035

Trial 2. Control (continued)

OBS	PCASHFE	PCASHMN	PCASHZN	KIDCA	KIDM	HCA	HMG	SCA	SMG	SHP	SC	IW	SW
1	0.0138	0.0039	0.0452	419	784	159	578	7.7	2.7	526	70	225	245
2	0.0059	0.0099	0.0441	393	664	127	565	8.2	3.3	490	68	237	264
3	0.0028	0.0046	0.0381	396	464	161	592	7.5	2.2	323	75	243	269
4	0.0090	0.0034	0.0345	438	499	140	573	8.3	2.5	328	51	263	283
5	0.0047	0.0033	0.0385	288	698	119	787	7.2	2.2	576	46	289	309
6	0.0170	0.0014	0.0433	415	723	140	579	7.8	1.4	453	61	310	329
7	0.0040	0.0186	0.0371	478	986	210	683	7.8	2.4	238	56	51	93
8	0.0014	0.0167	0.0360	519	971	210	688	7.6	2.8	140	58	55	95
9	0.0014	0.0219	0.0357	408	879	187	673	8.2	2.7	124	49	58	98
10	0.0173	0.0066	0.0352	412	897	231	744	8.2	2.3	4	62	55	107
11	0.0014	0.0263	0.0417	416	928	216	594	7.6	1.8	204	62	65	107
12	0.0012	0.0164	0.0357	487	988	189	714	7.4	2.1	84	50	65	103
13	0.0012	0.0242	0.0380	593	868	265	587	7.8	2.4	121	50	70	114

Trial 2. Experimental

OBS	AGE	CAL	PH	RG	RNO	AD	H_2O	VOLCC	GRGR	FFD	FFD/AD	ASH	ASH/AD	ASH/CC
1	Y	0.48	5.0	I	2	882	186	0.6956	1.2685	438	49.7	253	28.9	364
2	Y	0.48	5.0	J	16	806	210	0.5960	1.3523	395	49.1	224	27.8	376
3	Y	0.48	5.0	K	22	734	219	0.5153	1.4254	397	54.1	233	32.4	452
4	Y	0.48	5.0	L	39	815	257	0.5579	1.4618	419	51.4	258	31.7	463
5	Y	0.48	5.8	I	1	788	151	0.6365	1.2380	394	50.1	219	27.8	344
6	Y	0.48	5.8	J	17	857	252	0.6052	1.4171	449	52.4	261	30.5	431
7	Y	0.48	5.8	K	25	838	260	0.5785	1.4494	447	53.3	263	31.5	455
8	Y	0.48	5.8	L	38	835	261	0.5737	1.4558	433	51.9	246	29.5	431
9	Y	0.48	6.6	I	6	798	201	0.5973	1.3376	380	52.3	219	27.4	366
10	Y	0.48	6.6	J	18	832	226	0.6058	1.3745	459	55.2	268	32.2	442
11	Y	0.48	6.6	K	28	878	250	0.6277	1.3993	452	51.5	262	29.9	417
12	Y	0.48	6.6	L	33	840	269	0.5707	1.4720	455	54.2	266	31.7	466
13	Y	0.48	7.4	I	8	962	256	0.7067	1.3624	503	52.3	284	29.5	402
14	Y	0.48	7.4	J	15	814	224	0.5904	1.3794	436	53.6	250	30.8	424
15	Y	0.48	7.4	K	20	835	238	0.5971	1.3994	472	56.6	282	33.9	472
16	Y	0.48	7.4	L	35	745	237	0.5078	1.4683	419	56.2	248	33.3	488
17	Y	0.78	5.0	I	7	793	141	0.6523	1.2168	437	55.2	254	32.1	389
18	Y	0.78	5.0	J	12	706	191	0.5147	1.3717	382	54.2	222	31.5	431
19	Y	0.78	5.0	K	27	773	232	0.5408	1.4294	434	56.2	255	33.1	471
20	Y	0.78	5.0	L	37	879	285	0.5943	1.4796	464	52.8	272	31.0	458
21	Y	0.78	5.8	I	5	743	156	0.5870	1.2663	440	59.2	251	33.8	427
22	Y	0.78	5.8	J	14	815	222	0.5924	1.3758	431	52.9	259	31.8	437
23	Y	0.78	5.8	K	26	849	256	0.5922	1.4338	461	54.3	274	32.4	463
24	Y	0.78	5.8	L	32	797	270	0.5271	1.5122	427	53.7	252	31.7	478
25	Y	0.78	6.6	I	9	813	222	0.5909	1.3762	442	54.4	255	31.4	432
26	Y	0.78	6.6	J	19	815	214	0.6010	1.3564	448	55.0	262	32.2	437
27	Y	0.78	6.6	K	29	823	242	0.5808	1.4180	460	55.9	268	32.6	462
28	Y	0.78	6.6	L	31	771	234	0.5371	1.4364	439	57.0	266	34.5	495
29	Y	0.78	7.4	I	4	771	197	0.5741	1.3439	428	55.5	249	32.3	435
30	Y	0.78	7.4	J	11	806	227	0.5793	1.3927	450	55.8	269	33.4	465
31	Y	0.78	7.4	K	21	852	247	0.6050	1.4093	480	56.4	287	33.7	475
32	Y	0.78	7.4	L	34	826	271	0.5546	1.4903	474	57.4	288	34.9	519
33	M	0.48	5.0	M	1	767	193	0.5745	1.3365	571	74.5	350	45.6	609

Trial 2. Experimental (continued)

OBS	AGE	SAL	PH	RJ	RNO	AD	H_2O	VOLCC	SPGR	FPD	FPD/AD	ASH	ASH/AD	ASH/CC
34	M	0.48	5.0	N	19	779	196	0.5828	1.3368	564	72.4	358	46.1	615
35	M	0.48	5.0	O	25	787	196	0.5916	1.3317	559	71.0	351	44.6	594
36	M	0.48	5.0	P	32	833	204	0.6283	1.3259	579	69.6	370	44.4	589
37	M	0.48	5.8	M	8	767	174	0.5927	1.2941	559	71.7	346	45.1	583
38	M	0.48	5.8	N	17	837	204	0.6431	1.3232	578	69.1	363	43.4	573
39	M	0.48	5.8	O	27	802	226	0.5759	1.3940	555	69.2	352	43.9	611
40	M	0.48	5.8	P	33	874	220	0.6543	1.3370	659	75.4	424	48.5	649
41	M	0.48	6.6	M	6	763	184	0.5794	1.3176	563	73.8	357	46.8	616
42	M	0.48	6.6	N	11	848	214	0.6340	1.3386	614	72.4	395	46.6	623
43	M	0.48	6.6	O	20	806	199	0.6073	1.3286	564	70.0	353	43.8	581
44	M	0.48	6.6	P	38	927	251	0.6759	1.3725	642	69.3	409	44.2	606
45	M	0.48	7.4	M	5	800	181	0.6133	1.2924	569	71.1	374	46.7	604
46	M	0.48	7.4	N	16	890	242	0.6475	1.3751	621	69.8	388	43.7	600
47	M	0.48	7.4	O	28	923	271	0.6524	1.4157	638	69.1	406	44.0	623
48	M	0.48	7.4	P	35	837	228	0.6084	1.3757	580	69.3	369	44.2	607
49	M	0.78	5.0	M	4	650	150	0.4999	1.3007	474	72.9	298	45.8	596
50	M	0.78	5.0	N	14	811	221	0.5900	1.3758	580	71.6	371	45.8	629
51	M	0.78	5.0	O	22	831	245	0.5859	1.4190	590	71.0	376	45.3	642
52	M	0.78	5.0	P	37	892	256	0.6358	1.4037	642	71.9	402	45.1	632
53	M	0.78	5.8	M	3	761	173	0.5881	1.2954	563	73.9	363	47.7	617
54	M	0.78	5.8	N	18	790	234	0.5565	1.4206	553	70.0	351	44.5	631
55	M	0.78	5.8	O	26	881	250	0.6315	1.3959	649	73.7	411	46.6	650
56	M	0.78	5.8	P	30	855	249	0.6066	1.4107	602	70.4	383	44.9	632
57	M	0.78	6.6	M	7	846	221	0.6244	1.3552	622	73.6	397	47.0	636
58	M	0.78	6.6	N	12	879	221	0.6575	1.3374	628	71.5	417	47.5	635
59	M	0.78	6.6	O	24	839	240	0.5990	1.4007	584	69.6	366	43.6	611
60	M	0.78	6.6	P	29	843	255	0.5883	1.4336	609	72.2	387	46.0	658
61	M	0.78	7.4	M	9	815	210	0.6053	1.3476	587	72.0	375	46.1	620
62	M	0.78	7.4	N	15	919	293	0.6263	1.4681	651	70.9	416	45.3	664
63	M	0.78	7.4	O	21	786	175	0.6109	1.4068	585	74.5	394	50.2	646
64	M	0.78	7.4	P	34	949	278	0.6711	1.4152	667	70.3	421	44.4	628

Trial 1. Experimental (continued)

OBS	T	M	C	DA	FOA	CI	PCACHOA	PCAFHF	PCACHMA	PCACHMA	PCACHA	PCACHOM
1	3.225	2.125	1.100	4.68	56.6	0.3[illegible]	36.4	20.9	0.56	0.94	1.80	0.0007
2	3.085	2.030	0.995	3.94	55.0	0.329	35.[illegible]	21.4	0.54	1.14	2.[illegible]	0.[illegible]
3	3.090	2.050	1.040	4.19	56.0	0.337	37.9	22.[illegible]	0.[illegible]	1.11	2.32	0.[illegible]
4	2.940	1.950	0.990	3.90	56.0	0.337	3[illegible]	23.1	0.62	1.[illegible]	2.28	0.[illegible]
5	3.110	2.075	1.035	4.21	55.5	0.333	36.6	20.1	0.53	[illegible]	2.37	0.[illegible]
6	3.050	1.975	1.075	4.24	58.1	0.352	38.4	21.3	0.63	[illegible]	2.48	0.[illegible]
7	3.025	1.985	1.040	4.09	56.9	0.344	38.0	22.4	0.59	1.34	2.[illegible]	0.[illegible]
8	3.110	2.000	1.110	4.45	58.6	0.357	37.4	21.8	0.58	1.16	1.95	0.[illegible]
9	3.160	2.100	1.060	4.37	55.8	0.335	39.6	21.7	0.53	0.93	1.41	0.[illegible]
10	3.180	2.125	1.055	4.39	55.3	0.332	39.7	23.4	0.57	1.34	2.7[illegible]	0.[illegible]
11	3.150	2.000	1.150	4.64	59.7	0.365	37.2	21.[illegible]	0.[illegible]	1.21	2.34	0.[illegible]
12	2.985	1.930	1.055	4.07	58.2	0.353	38.7	22.0	0.58	1.32	2.03	0.[illegible]
13	3.150	2.075	1.075	4.40	56.6	0.341	37.0	21.5	0.55	1.11	2.[illegible]	0.[illegible]
14	3.190	2.110	1.080	4.49	56.2	0.339	38.9	22.3	0.56	1.16	2.57	0.[illegible]
15	3.275	2.050	1.225	5.12	60.8	0.374	37.8	21.[illegible]	0.62	1.22	2.14	0.[illegible]
16	2.950	1.890	1.060	4.02	59.0	0.359	36.5	20.9	0.48	1.13	2.56	0.[illegible]
17	3.020	2.050	0.970	3.86	53.9	0.321	37.7	21.2	0.54	0.95	1.40	0.[illegible]
18	3.070	2.090	0.980	3.97	53.7	0.319	38.4	21.5	0.55	1.06	1.97	0.[illegible]
19	3.010	2.040	0.970	3.84	54.1	0.322	38.1	22.9	0.50	1.[illegible]	2.34	0.[illegible]
20	3.125	2.020	1.105	4.46	58.2	0.354	36.0	20.5	0.55	1.03	1.70	0.[illegible]
21	3.310	2.260	1.050	4.59	53.4	0.317	37.7	20.8	0.58	0.93	1.96	0.0023
22	3.190	2.100	1.090	4.52	56.7	0.342	39.2	22.6	0.60	1.28	1.93	0.0037
23	3.055	2.025	1.030	4.10	56.1	0.337	39.0	22.5	0.58	1.17	1.47	0.0021
24	3.045	1.910	1.135	4.41	60.7	0.373	38.3	21.9	0.51	1.33	1.94	0.0046
25	3.025	1.875	1.150	4.42	61.6	0.380	38.9	22.5	0.57	1.06	1.11	0.0016
26	3.245	2.180	1.065	4.53	54.9	0.328	38.7	21.8	0.56	1.17	2.27	0.0055
27	2.975	1.895	1.080	4.12	59.4	0.363	37.9	22.0	0.53	1.23	2.11	0.0046
28	3.230	2.110	1.120	4.69	57.3	0.347	36.4	20.9	0.49	1.10	1.95	0.0039
29	2.950	1.850	1.100	4.14	60.7	0.373	41.7	24.4	0.62	1.11	2.04	0.0052
30	3.270	2.200	1.070	4.59	54.7	0.327	38.5	21.3	0.52	0.92	0.75	0.0007
31	3.030	1.860	1.170	4.49	62.3	0.386	36.5	21.7	0.54	1.25	2.57	0.0042
32	3.175	1.980	1.195	4.83	61.1	0.376	38.1	22.0	0.59	1.29	2.04	0.0007
33	3.075	1.755	1.320	5.00	67.4	0.429	40.8	23.4	0.72	1.45	1.59	0.0060

Trial 2. Experimental (continued)

OBS	T	M	C	CA	PCA	CI	PCASHCA	PCASHP	PCASHMG	PCASHNA	PCASHK	PCASHCL
34	3.360	2.085	1.275	5.45	61.5	0.379	40.2	22.4	0.68	1.51	1.48	0.0050
35	3.390	2.070	1.320	5.65	62.7	0.389	40.0	22.7	0.68	1.51	1.47	0.0051
36	3.575	2.350	1.225	5.69	56.4	0.343	39.7	21.9	0.71	1.21	0.79	0.0039
37	3.240	1.940	1.300	5.55	67.4	0.429	38.5	22.5	0.66	1.39	1.08	0.0045
38	3.255	1.935	1.320	5.37	64.7	0.406	39.3	21.8	0.67	1.14	0.97	0.0039
39	3.510	2.255	1.225	5.68	58.7	0.358	39.5	22.1	0.67	1.30	1.38	0.0057
40	3.650	2.305	1.345	6.28	60.1	0.368	38.6	22.0	0.67	1.39	1.40	0.0038
41	3.245	1.950	1.295	5.28	63.9	0.399	40.5	22.6	0.71	1.57	1.38	0.0062
42	3.470	2.150	1.320	5.82	61.6	0.380	40.9	22.3	0.71	1.19	0.69	0.0030
43	3.550	2.260	1.290	5.98	59.5	0.363	40.5	22.2	0.66	1.15	0.99	0.0040
44	3.530	2.005	1.525	6.62	67.7	0.432	39.2	22.0	0.73	1.40	1.38	0.0048
45	3.475	2.100	1.375	6.01	63.5	0.396	40.0	23.1	0.65	1.56	2.07	0.0072
46	3.520	2.060	1.460	6.39	65.8	0.415	38.3	21.8	0.65	1.28	1.25	0.0046
47	3.665	2.375	1.290	6.11	58.0	0.352	39.7	22.2	0.69	1.44	1.22	0.0015
48	3.545	2.155	1.390	6.22	63.0	0.392	38.3	21.2	0.54	1.22	1.09	0.0046
49	2.865	1.645	1.220	4.31	67.0	0.426	42.1	23.6	0.68	1.37	1.47	0.0060
50	3.420	2.250	1.170	5.20	56.7	0.342	40.0	22.2	0.71	1.36	1.29	0.0048
51	3.625	2.250	1.375	6.34	61.5	0.379	38.6	21.7	0.65	1.22	1.22	0.0037
52	3.640	2.225	1.415	6.51	62.6	0.389	38.4	21.6	0.65	1.21	1.08	0.0033
53	3.305	2.010	1.295	5.40	63.0	0.392	39.3	22.1	0.61	1.39	1.51	0.0055
54	3.315	2.010	1.305	5.45	63.2	0.394	39.2	21.5	0.61	1.20	0.92	0.0046
55	3.495	2.150	1.345	5.96	62.2	0.384	38.3	22.1	0.66	1.24	0.90	0.0039
56	3.460	2.200	1.260	5.59	59.6	0.364	37.9	21.1	0.64	1.23	1.11	0.0036
57	3.490	2.110	1.380	6.06	63.4	0.395	40.4	23.2	0.73	1.45	1.55	0.0050
58	3.600	2.190	1.410	6.40	63.0	0.392	39.9	22.3	0.63	1.34	0.97	0.0037
59	3.440	2.125	1.315	5.74	61.8	0.382	39.5	21.8	0.67	1.29	1.10	0.0049
60	3.315	2.010	1.305	5.45	63.2	0.394	40.7	23.3	0.75	1.59	1.60	0.0062
61	3.440	2.040	1.400	6.02	64.8	0.407	40.6	23.4	0.73	1.58	1.58	0.0059
62	3.610	2.230	1.380	6.32	61.8	0.382	39.6	22.6	0.66	1.36	1.35	0.0038
63	3.440	2.120	1.320	5.76	62.0	0.384	39.0	21.7	0.64	1.26	1.06	0.0052
64	3.560	2.125	1.435	6.40	64.4	0.403	37.8	21.2	0.65	1.33	0.96	0.0038

Trial 2. Experimental (continued)

OBS	PCASHFE	PCASHMN	PCASHZN	KIDCA	KIDN	HCA	HMG	SCA	SMG	SHP	SC	IW	SW	RW
1	0.0007	0.0086	0.0336	19103	1067	209	837	9.0	3.6	612	59	62	91	232
2	0.0016	0.0014	0.0376	1779	938	204	797	8.0	3.8	676	62	62	100	224
3	0.0039	0.0139	0.0380	21297	1025	218	756	9.6	3.8	722	59	67	107	235
4	0.0126	0.0037	0.0451	21276	1130	226	829	8.1	4.1	702	53	76	117	221
5	0.0008	0.0056	0.0345	7342	901	220	909	8.2	3.3	604	53	61	91	243
6	0.0014	0.0089	0.0377	39715	1414	165	782	9.2	3.9	656	54	61	103	250
7	0.0116	0.0129	0.0503	38345	1017	217	869	8.5	3.5	702	56	60	108	250
8	0.0022	0.0130	0.0340	34756	1358	165	803	8.4	3.8	670	61	66	115	236
9	0.0009	0.0119	0.0329	65361	1778	157	833	8.6	2.9	604	52	49	95	210
10	0.0052	0.0022	0.0380	77949	1482	197	777	9.9	4.3	672	56	65	104	232
11	0.0025	0.0017	0.0394	93576	2480	171	784	8.7	4.1	616	73	62	106	250
12	0.0023	0.0135	0.0353	93444	2466	183	943	7.7	2.9	642	54	59	112	236
13	0.0104	0.0081	0.0367	74313	1897	147	827	9.2	3.9	596	44	55	95	225
14	0.0011	0.0089	0.0567	43923	1417	159	827	7.8	3.2	596	43	58	99	230
15	0.0092	0.0079	0.0488	25356	1243	192	813	8.0	3.6	664	49	54	105	259
16	0.0056	0.0089	0.0410	49803	1529	166	954	8.7	3.1	576	51	67	113	246
17	0.0008	0.0008	0.0482	10073	867	297	765	9.0	3.4	644	48	52	95	203
18	0.0009	0.0135	0.0368	11810	819	226	728	8.7	3.3	684	44	53	97	203
19	0.0039	0.0141	0.0383	2622	747	223	692	8.3	2.8	604	48	61	108	242
20	0.0066	0.0117	0.0315	2595	878	200	751	8.5	3.5	710	51	62	115	233
21	0.0099	0.0084	0.0367	30872	1480	192	785	8.7	3.5	646	49	54	94	210
22	0.0007	0.0015	0.0545	14349	533	257	808	8.0	2.9	644	45	55	99	231
23	0.0028	0.0007	0.0428	32750	1219	171	808	9.2	3.8	604	51	60	108	236
24	0.0008	0.0137	0.0357	41109	1345	246	839	7.7	2.6	628	53	64	111	213
25	0.0277	0.0122	0.0383	551	891	147	813	7.7	4.1	604	50	56	96	214
26	0.0032	0.0142	0.0380	5824	691	221	824	7.6	3.3	654	58	56	104	239
27	0.0023	0.0015	0.0356	74892	1835	179	787	8.3	3.7	596	56	64	108	234
28	0.0016	0.0133	0.0359	80133	1774	239	853	8.8	3.7	568	52	61	108	230
29	0.0164	0.0138	0.0371	41357	1286	183	783	8.8	3.4	596	47	55	93	200
30	0.0007	0.0067	0.0311	26237	978	168	836	8.1	3.0	630	42	57	96	220
31	0.0076	0.0028	0.0382	29383	997	184	772	8.6	3.3	632	43	64	105	218
32	0.0035	0.0125	0.0479	4379	967	176	942	7.7	2.9	556	51	64	112	240
33	0.0109	0.0049	0.0393	415	799	205	812	7.9	3.8	550	62	216	234	268

Trial 2. Experimental (continued)

OBS	PCASHFE	PCASHMN	PCASHZN	KIDCA	KIDM	HCA	HMG	SCA	SMG	SHF	SC	IW	SW	RW
34	0.0162	0.0050	0.0412	421	656	187	876	8.6	3.2	424	52	253	278	320
35	0.0074	0.0080	0.0415	406	710	276	894	9.3	2.8	422	72	269	286	321
36	0.0099	0.0049	0.0474	456	651	208	925	8.4	2.4	404	53	290	310	328
37	0.0150	0.0029	0.0451	448	756	192	876	7.9	3.6	452	68	235	257	287
38	0.0088	0.0050	0.0407	397	726	197	842	7.3	2.7	434	68	257	275	297
39	0.0148	0.0074	0.0392	365	659	222	864	7.9	3.0	418	66	275	290	321
40	0.0090	0.0005	0.0457	443	692	230	986	7.9	2.6	436	57	298	310	319
41	0.0134	0.0056	0.0437	390	737	173	971	8.1	3.4	426	72	232	250	282
42	0.0101	0.0035	0.0395	444	743	198	850	8.5	3.1	416	60	240	265	281
43	0.0119	0.0028	0.0430	376	776	241	898	8.8	3.1	412	62	258	279	308
44	0.0122	0.0063	0.0464	327	730	177	909	8.4	3.2	432	61	312	338	352
45	0.0253	0.0096	0.0476	388	717	220	943	7.7	2.6	378	47	227	250	278
46	0.0113	0.0036	0.0437	311	718	181	924	9.1	3.7	404	57	253	274	298
47	0.0128	0.0005	0.0447	377	761	142	926	7.5	3.2	404	57	275	296	318
48	0.0029	0.0052	0.0336	368	804	218	889	7.9	1.9	432	59	302	318	296
49	0.0174	0.0034	0.0456	491	675	223	811	7.1	3.3	416	49	227	245	269
50	0.0065	0.0059	0.0425	426	789	209	863	9.3	3.7	384	53	245	270	315
51	0.0069	0.0005	0.0467	422	550	295	849	8.1	2.9	496	53	262	280	299
52	0.0052	0.0033	0.0418	411	520	242	841	8.3	2.8	470	54	313	335	373
53	0.0086	0.0074	0.0399	414	712	192	822	8.5	3.4	390	52	218	239	280
54	0.0137	0.0034	0.0472	428	650	210	902	9.9	3.0	464	60	257	277	291
55	0.0092	0.0049	0.0404	405	744	218	862	8.4	2.6	370	67	269	287	319
56	0.0057	0.0031	0.0443	416	697	266	884	7.7	3.1	444	60	279	305	342
57	0.0151	0.0050	0.0478	345	731	225	920	8.4	3.1	396	62	232	257	287
58	0.0112	0.0027	0.0415	458	790	219	838	9.1	3.1	400	64	242	268	295
59	0.0076	0.0066	0.0470	401	743	178	862	8.5	3.3	412	57	265	286	313
60	0.0114	0.0057	0.0516	376	629	175	916	7.8	2.4	396	69	277	300	320
61	0.0138	0.0048	0.0522	350	947	188	857	8.0	3.3	372	53	236	262	288
62	0.0135	0.0029	0.0457	381	611	194	905	8.4	3.1	424	47	247	271	299
63	0.0046	0.0034	0.0521	380	628	198	900	8.1	3.2	392	47	261	280	306
64	0.0043	0.0024	0.0427	361	779	205	938	8.3	1.4	364	52	300	312	319